The Impact of Toll-like Receptor Signaling on Murine Herpesvirus 68 Infection *In Vitro* and *In Vivo*

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von

Florian Haas

aus

Basel

Promotionskomitee

Prof. Dr. David Nadal (Vorsitz und Leitung der Dissertation)

Prof. Dr. Mathias Ackermann

Prof. Dr. Hans-Peter Beck

Prof. Dr. Cornel Fraefel

Prof. Dr. Urs F. Greber

Prof. Dr. Roberto F. Speck

Dr. Michele Bernasconi

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Florian Haas

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Summary

Epstein-Barr virus (EBV) infects an estimated 95% of the adult population and establishes a life-long persistent infection in B-Lymphocytes. While primary infection is usually without symptoms, latent infection with EBV is associated with Burkitt's Lymphoma (BL) and several other forms of B-cell malignancies that are a challenge for medicine. The evident association of Burkitt's lymphoma with *Plasmodium falciparum* infection suggests a role for co-infections in the etiology of BL, even though the exact mechanism is not known to date. Chronic infection with *P. falciparum* causes strong stimulation of innate immune pathways by triggering germ-line encoded pattern-recognition receptors such as Toll-like receptors (TLRs). Importantly, stimulation of TLR9 signaling has been shown to have a direct impact on EBV gene expression and reactivation, providing a possible connection between *P. falciparum* and malignancies arising from latently EBV-infected B-cells.

In the work presented here, we used murine gamma-herpesvirus 68 (MHV-68) to investigate the impact of endosomal TLRs 7 and 9 on gamma-herpesvirus reactivation and establishment of latency. The use of MHV-68 allowed us to not only study endosomal TLR triggering *in vitro*, but also assess the consequences of repeated TLR stimulation *in vivo*. We found that stimulation of TLR7 and TLR9 suppresses lytic MHV-68 gene expression via activation of the cellular transcription factor NF κB, therefore maintaining the virus in the latent state. In immune competent hosts, the repeated administration of TLR7 ligand was found to augment the frequency of latently infected cells, indicating that constant stimulation of innate immune receptors promote MHV-68 latency.

Our results demonstrate that a pathway of the innate immune system suppresses MHV-68 lytic replication via activation of NF κB and favours the establishment of latent infection. This provides insight into a possible role for co-infections in the etiology of gamma-herpesvirus-associated malignancies and will hopefully open up new avenues for understanding and preventing these cancers.

Zusammenfassung

Das Epstein-Barr Virus (EBV) infiziert geschätzte 95% der erwachsenen Weltbevölkerung und etabliert eine lebenslang persistierende Infektion in B-Lymphozyten. Während die Primärinfektion üblicherweise ohne Symptome verläuft, ist latente EBV Infektion mit Burkitt's Lymphomen und anderen Formen von bösartigen B-Zell Tumoren assoziiert, die eine Herausforderung für die Medizin darstellen. Die erwiesene Assoziation von Burkitt's Lymphomen mit *Plasmodium falciparum* deutet darauf hin, dass Co-Infektionen bei der Entstehung von BL eine Rolle spielen, auch wenn der genaue Mechanismus bis heute unbekannt ist. Durch das Auslösen von Rezeptoren, die körperfremde Muster erkennen wie zum Beispiel Toll-like Rezeptoren (TLRs) verursacht chronischer Befall mit *P. falciparum* eine starke Stimulierung von Signalwegen des angeborenen Immunsystems. Es wurde gezeigt, dass die Stimulierung von TLR9 einen direkten Einfluss auf EBV Genexpression und Reaktivierung hat, was eine mögliche Verbindung zwischen *P. falciparum* und dem Auftreten von Tumoren aus EBV-infizierten B-Zellen darstellt.

In der hier vorliegenden Arbeit haben wir einen murinen gamma-Herpesvirus (MHV-68) verwendet, um den Einfluss der endosomalen TLRs 7 und 9 auf gamma-Herpesvirus Reaktivierung und Etablierung der Latenz zu untersuchen. Die Verwendung von MHV-68 erlaubt es, nicht nur den Effekt endosomaler TLR Signale *in vitro* zu studieren, sondern auch die Konsequenzen wiederholter TLR Stimulierung *in vivo*. Wir haben gezeigt, dass Stimulierung von TLR7 und 9 die Expression von lytischen MHV-68 Genen durch die Aktivierung des zellulären Transkriptionsfaktors NF κB unterdrückt und das Virus dadurch im Zustand der Latenz hält. Im immunkompetenten Wirt führte die wiederholte Zugabe von TLR7 Ligand zu einer erhöhten Frequenz an latent infizierten Zellen, was darauf hindeutet, dass konstante Stimulierung von Rezeptoren des angeborenen Immunsystems latente Infektion von MHV-68 fördert.

Unsere Resultate zeigen, dass ein Signalweg des angeborenen Immunsystems die Fähigkeit hat, die lytische Replikation von MHV-68 durch Aktivierung von NF κB zu unterdrücken, was die Etablierung einer latenten Infektion begünstigt. Diese Erkenntnis bietet einen Anhaltspunkt für die mögliche Rolle von Co-Infektionen bei der Entstehung von gamma-Herpesvirus-assoziierten Tumoren und wird hoffentlich neue Wege aufzeigen diese Tumore zu verstehen und zu verhindern.

Introduction

Epstein-Barr Virus and associated lymphoma

Epstein-Barr virus (EBV) is a B-lymphotropic virus belonging to the subgroup of gamma-herpesviruses. The virus is very widespread in the adult population with an estimated 95% of the people being seropositive. Despite the infection being generally without symptoms, EBV poses a great challenge for modern medicine due to the association of latent EBV infection with a range of malignant lymphomas in human patients.

Primary infection with EBV generally occurs during infancy and is without symptoms in the vast majority of cases. Characteristically for all herpesviruses, EBV establishes a latent infection in the host with intermittent periods of active replication leading to the production of viral progeny necessary for transmission. Co-evolution of virus and host has established an intricate equilibrium where the virus persists in the host at low levels of infection without being cleared by the immune system and without apparent harm to the host.

EBV was the first human gamma-herpesvirus detected and was originally described in 1964 by Anthony Epstein, who identified viral particles in samples of Burkitt's Lymphoma from Uganda [1]. EBV was the first virus to be identified as a potential tumorigenic agent and EBV infection was subsequently found to be associated with other B-cell malignancies such as Hodgkin's disease [2] or post-transplant lymphoproliferative disorders [3]. EBV infection is also associated with non-B-cell tumors such as nasopharyngeal carcinoma [4] where EBV latency genes are found in the majority of cases [5] and is also found in 10% of all gastric carcinomas [6]. The tumorigenicity of EBV is confirmed experimentally by the fact that infection with EBV is sufficient to growth-transform human B-lymphocytes *in vitro* [7]. A second member of the human gamma-herpesvirus family was detected in 1994 in Kaposi's sarcoma tissue obtained from patients with acquired immunodeficieny syndrome (AIDS) and was termed Kaposi's sarcoma-associated Herpesvirus (KSHV) [8]. The same viral entity was found to be associated with lymphoproliferative disorders such as Multicentric Castleman's Disease [9] and primary effusion lymphoma (PEL) [10], indicating that tumorigenicity is not a trait exclusive to EBV but might be a feature common to all gamma-herpesviruses.

EBV-associated lymphoma are relatively rare considering a majority of adults is latently infected. It has therefore become a critical challenge to identify the mechanisms of gamma-herpesvirus-associated lymphomagenesis and the physiological circumstances that predispose an individual to EBV-associated malignancies. Some of these malignancies occur in patients where the immune system is weakened due to immunosuppressive treatment after

organ transplantation or advanced stages of acquired immunodeficiency syndrome (AIDS) caused by infection with human immunodeficiency virus (HIV). These incidents of EBV-positive lymphoma are likely explained by the deteriorating immune control of latently infected B-cells through cytotoxic immune cells and underscore the importance of constant immune supervision to balance asymptomatic latent infection.

However, EBV-positive lymphoma also occur in immunocompetent patients, the prime example being endemic Burkitt's Lymphoma (eBL), the tumour entity in which EBV was first identified. Notably, these tumours occur almost exclusively in areas where the causative agent of malaria, *Plasmodium falciparum* is holoendemic [11,12], demonstrating a role for co-infections in the ontology of EBV-associated lymphoma in immunocompetent patients that is unclear so far.

Unfortunately, the study of EBV infection and associated lymphomagenesis in the context of an intact immune system is hampered by the strict species specificity of EBV, therefore, a robust animal model is vital to understand the interplay between virus and host immunity. Murine gamma-herpesvirus 68 (MHV-68) is widely used as a model to investigate gamma-herpesvirus infection *in vivo* as it readily infects common strains of laboratory mice. MHV-68, which was originally isolated from free-living wood mice, is genetically closely related to EBV and shares important features of its biology, including types of infection (productive replication and latency), primary site of latent infection (B-cells) and associated diseases (lymphoproliferative malignancies). By studying MHV-68 infection in mice, we have gained a lot of insight about gamma-herpesvirus biology in general, from the course of initial infection and the establishment of latency to host immune response and viral immune evasion.

MHV-68: a model for gamma-herpesvirus infection *in vivo*

My work described in this thesis focuses heavily on using MHV-68 infection of laboratory mice to study gamma-herpesvirus biology both *in vitro* and *in vivo*. In order to evaluate the results of my study and put it in the proper context, I have written a review that summarizes the knowledge gained about gamma-herpesvirus infection *in vivo* using this system and that is currently in preparation for submission (see below). It serves to illustrate the possibilities, but also the limitations of the system with respect to investigating EBV-associated lymphomagenesis, as is known up to now.

Origin of MHV-68 and natural host reservoir

How MHV-68 was first isolated and introduced into experimental animals is rather intriguing and important for the characteristics exhibited during infection. Five different strains of a herpesvirus were isolated from two different free-living small rodent species (*Apodemus flavicollis* and *Clethrionomys glareolus)* during field studies in Slovakia in 1980 [13]. It was concluded that these viruses were probably geographically widespread throughout mouse and vole subfamilies. Noteworthy, all five strains were able to replicate in fibroblast cell cultures from various animal species (birds, rodents, pigs, monkey) and man, which is interesting since gamma-herpesviruses normally have a very narrow range of host specificity. Based on the growth characteristics and cytopathic changes typical for members of the herpesvirus family all five murid herpesvirus 4 isolates were originally classified as *Alphaherpesvirinae* [14].

Subsequent field studies in the UK found that 13-24% of wood mice (*Apodemus sylvaticus*) have serum antibodies reactive to MuHV-4 contrasting 3% of bank voles (*Clethrionomys glareolus*) and confirmed the presence of MuHV-4 DNA in the lungs and spleens of infected animals. This suggests that wood mice are indeed the natural host rather than voles [15,16]. In free-living animals of the species used for laboratory work (*Mus musculus*), MuHV-4 was very rarely detected [17], indicating that *Mus musculus* is only an accidental host and prompting the question whether an infection of laboratory mice reflects the natural course of infection. Indeed, the course and characteristics of MHV-68 infection in wood mice differ from those in the commonly used laboratory experimental mouse strain BALB/c in terms of titers during acute infection in the lungs as well as some features of viral immune evasion. Nevertheless, the overall characteristics of gamma-herpesvirus infection seem to be present in laboratory mice [18,19]. Therefore, infection of mice with MuHV-4 is a promising model system to study gamma-herpesvirus biology under controlled laboratory conditions and the presence of an intact immune system enables us to investigate the role of immune stimulation. Additionally, the availability of genetically engineered mouse strains provides a powerful tool to study the interaction of gamma-herpesviruses with specific parts of the host immune system in order to learn how chronic infection is controlled *in vivo*.

Classification and genetic relation to human herpesviruses

Sequence analysis of restriction fragments revealed that MHV-68 is more closely related to gamma-herpesviruses than to the *Alphaherpesvirinae* as originally classified based on *in vitro* culture characterization [20]. Further molecular studies showed that the MHV-68 genome

consists of a singular stretch of 118kb unique DNA sequence flanked by a variable number of terminal repeats. This genomic structuring is strikingly similar to that of a subgroup of gamma-herpesviruses termed gamma-2 or *Rhadinovirus*, comprising herpesviruses from New World monkeys such as herpesvirus saimiri (HVS) and herpesvirus ateles (HVA) but also human pathogenic KSHV [21]. The other subgroup of gamma-herpesviruses termed gamma-1 or *Lymphocryptovirus*, comprise human pathogenic EBV and several simian herpesviruses detected in Old World non-human primates (Fig. 1). In contrast to the gamma-2 herpesviruses, they have a coding region divided into two segments (small and large unique region) divided by internal repeated regions and flanked by terminal repeats [22]. Based on these characteristics, MHV-68 was classified as belonging to the order *Herpesvirales*, family *Herpesviridae*, subfamily *Gammaherpesvirinae*, genus *Rhadinovirus* and species *Murid Herpesvirus 4* [23]. Phylogenetic analysis suggests that MuHV-4 and primate rhadinoviruses diverged about 60 million years ago [24].

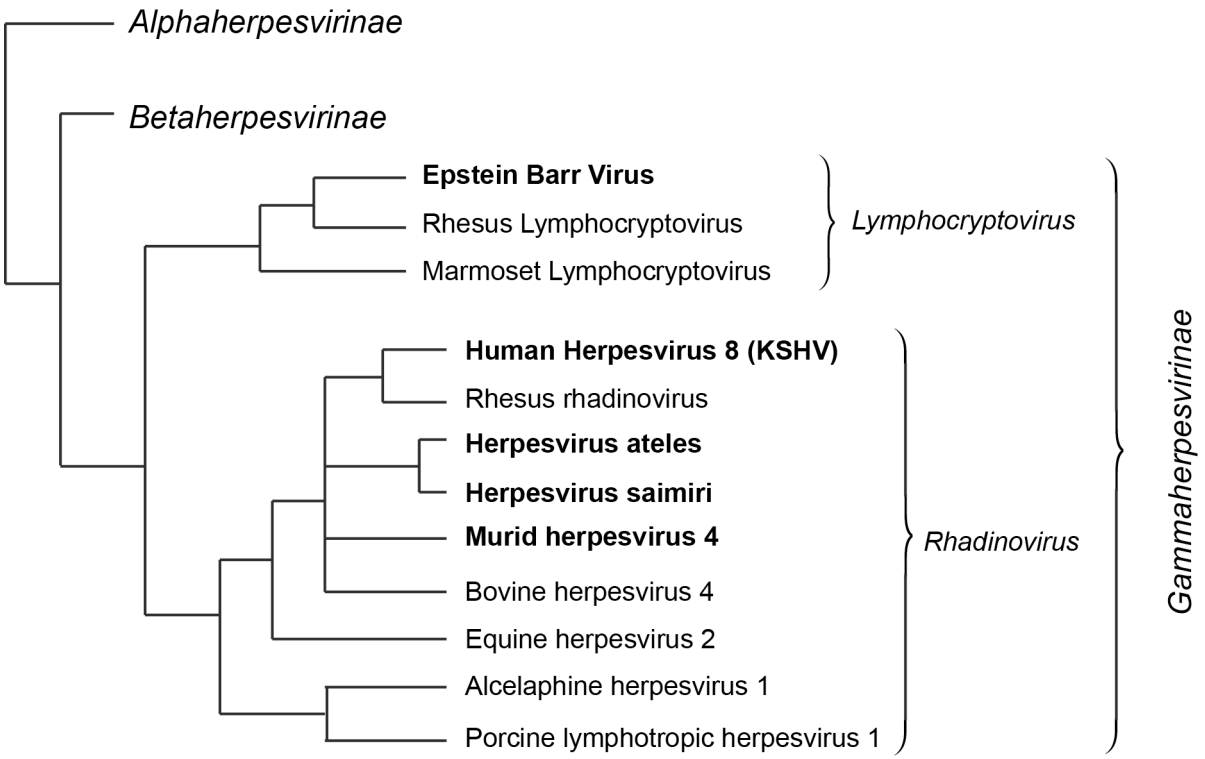


Figure 1. Phylogenetic tree for the *Gammaherpesvirinae* showing the two distinct subgroups, *Lymphocryptovirus* (gamma-1-herpesvirus) and *Rhadinovirus* (gamma-2-herpesvirus) (adapted from DJ McGeoch et al. [25]). Species mentioned in the text are shown in bold.

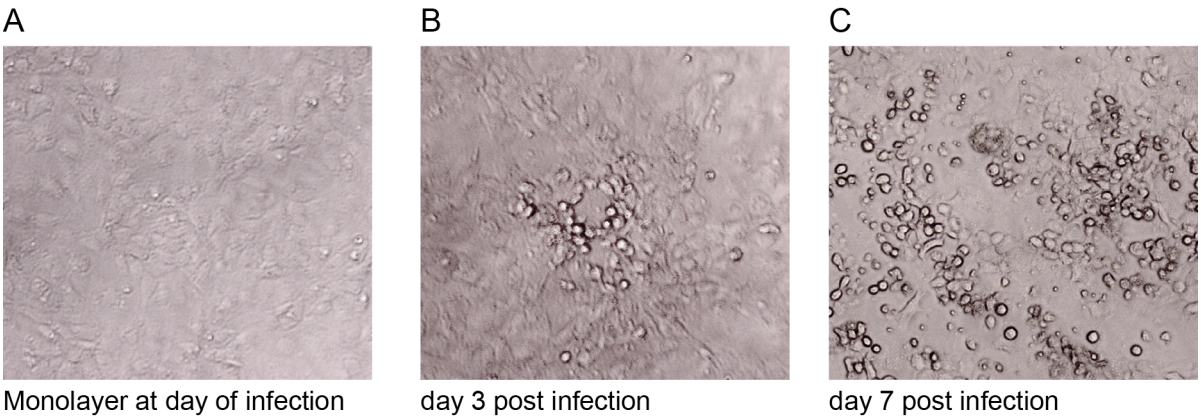
Complete sequencing of the MHV-68 genome predicted at least 80 open reading frames (ORFs), 63 of which are co-linear and homologous to rhadinoviruses HVS and KSHV and many of which are also present in the EBV genome [26]. The considerable amount of

conservation is a strong indication of common mechanisms for host colonization and regulation of the viral life cycle, which is a prerequisite to use MHV-68 infection as a model system for gamma-herpesviruses. However, interspersed in between these conserved blocks of genes, which seem to be common to all gamma-herpesviruses, each virus contains a number of unique genes, which may reflect host-species-specific adaptations or constraints. MHV-68 contains 14 ORFs, which have no apparent homologs in HVS, KSHV, or EBV and seem to be specific for this particular virus. Notably, none of the EBV latency-associated genes that have been shown to be essential for transformation of B-cells *in vitro* (LMP1, EBNAs) appear to have any homologs in the other gamma-herpesviruses. This in turn means that while MHV-68 and EBV share large homology in their genomes and therefore have probably many common features, there are also differences affecting the maintenance of latent infection and possibly the oncogenic potential of the virus.

MHV-68 infection *in vitro*

As mentioned above, MHV-68 readily infects fibroblast cell lines of various origin [14]. In these cells, MHV-68 replicates with concurrent expression of most viral genes, leading to cytopathic effects (CPE) (Fig. 2) and accumulation of high titers of infectious viral particles in the supernatant, similar to human rhadinovirus KSHV, that causes CPE and replicates in human embryonal-kidney epithelial cells 293 [27]. In contrast, the infection of epithelial cells *in vitro* by cell free EBV is very inefficient and seems to require the presence of infected B-cells in a transfer infection assay [28].

Figure 2. Lytic infection of mouse fibroblast cell line NIH3T12 with MHV-68 in vitro. NIH3T12 cells were



grown to a monolayer and then infected with MHV-68 at a multiplicity of infection of 0.1. A) At the day of infection, fibroblasts are forming a tight monolayer. B) 3 days post infection, foci of detached cells have appeared, forming holes in the monolayer and indicating cytopathic effect through lytic viral replication. C) 7 days post infection, initial infection has spread and a majority of fibroblasts have detached from the plate.

In vivo, B-lymphocytes are thought to be the major reservoir of gamma-herpesvirus latency, therefore considerable effort has been put into studying infection of B-lymphocytes *in vitro*. Indeed, it was found that MHV-68 persistently infects mouse myeloma cells NSO (B-cells) but not mouse thymoma cells BW5147 (T-cells) [29]. Infection of primary murine B-lymphocytes *in vitro* leads to activation, proliferation and prolonged survival of these cells. However, there is no evidence of productive MHV-68 replication and the viral genome is found predominantly in a linear form instead of the circularized genome typical for viral latency [30]. This suggests that MHV-68 enters primary B-lymphocytes *in vitro* and that the viral genome is successfully uncoated, but persistent infection or growth-transformation is not achieved. Again, this is in sharp contrast with EBV, where infection of primary B-lymphocytes *in vitro* is very efficient and growth-transformation leads to the outgrowth of persistently infected lymphoblastoid cell lines [7]. It is likely that the contrasting results of *in vitro* infection reflect the differences in latency genes being expressed in the host cell and highlights the importance of the EBV latency genes for efficient transformation.

Course of infection in immunocompetent mice

Even though field studies suggest that laboratory mice (*Mus musculus*) are not the natural host for MHV-68 (see above), the virus exhibits a course of infection typical for gamma-herpesviruses in immunocompetent inbred mouse strains by establishing a persistent life-long infection without apparent signs of disease. Nonetheless, one of the unresolved questions of the MHV-68 mouse model is how the virus manages to colonize its host. Despite efforts to study transmission from infected to naive mice, the route of initial infection is still somewhat unclear. There have been reports of vertical transmission from chronically infected females to newborn pups [31] either via transplacental transmission or via shedding of infectious particles in breast milk similar to the beta-herpesvirus murine Cytomegalovirus (mCMV) [32] or the human virus KSHV [33]. Indeed, the gastrointestinal tract seems to fulfill the requirements of a portal of entry, since oral administration or gastric instillation of MHV-68 can lead to the establishment of latent infection in the spleen [34]. Any attempts to study horizontal transmission within a population have been largely unsuccessful. Therefore, it is unclear how MHV-68 spreads under natural circumstances. A recent study reports MHV-68 shedding by genital excretion and transmission from infected females to naive males via sexual contact [35], a route that is also used by Herpes simplex virus (HSV) type 2 [36]. Finally, the detection of MHV-68 DNA in ticks (*Ixodes ricinus*) by polymerase chain reaction

raises the possibility of a role for arthropode vectors in transmission of MHV-68 in nature [37].

Human gamma-herpesviruses are mainly transmitted via airborne infection and colonize the new host via airway epithelia, and shedding of virus into saliva of infectious patients have been shown for both EBV and KSHV [38-40]. In an attempt to model natural gamma-herpesvirus infection, the most common technique of experimental infection is therefore intranasal inoculation to allow access to the epithelium of the upper respiratory tract. The respiratory tract likely represents a natural route of infection, but experimental *i.n.* inoculaton of MHV-68 is not without pitfalls. Although *i.n.* inoculation of MHV-68 has to be done under general anesthesia, this does not ensure an equal infectious dose due to unknown amounts of inoculum being swallowed. As an alternative, intra-peritoneal (i.p.) injection of MHV-68 allows direct seeding to the major reservoir of latent infection, i.e. the spleen [41]. This results in robust latent infection of splenocytes and establishes long-term infection in peritoneal macrophages [42]. Different infection techniques may present advantages in specific experimental settings, nevertheless, intranasal inoculation is most widely used today.

After intranasal inoculation, replicating MHV-68 can be detected in the lungs [43]. The main site of this acute replication phase following primary infection seems to be alveolar epithelium, where MHV-68 replicates to high titers during the first three days. The acute infection phase is terminated by a robust immune response in immunocompetent mice as macrophages and mononuclear cells, predominantly CD8+ T-cells, infiltrate the lungs and infectious virus is largely cleared 7-10 days post inoculation. Acute viral replication in the respiratory tract generally takes place without clinical symptoms but may lead to severe disease that can even be fatal in some cases [43].

In a second phase, MHV-68 eventually gains entry to the lymphoid compartment, most likely by infecting B-lymphocytes in the local mediastinal lymph nodes (MLN). Evidence suggests that dendritic cells (DCs) play an important role in delivering virus to the MLN. The use of a recombinant marker virus revealed that a significant fraction of virus found in B-cells bore genetic marks from an earlier passage in dendritic cells and a virus attenuated for replication in DCs showed severe impairment in B-cell colonization [44]. This suggests that MHV-68 uses DCs to bridge the gap from initial replication in alveolar epithelium to reaching the MLN and thus gaining access to the lymphoid compartment. From the MLN, MHV-68 spreads to the spleen and other lymphoid tissues, presumably via infected circulating B-lymphocytes. This is suggested by the observation that B-cell deficient mice exhibit strong impairment of

latency establishment in the spleen following intranasal inoculation despite normal titers in the lung [45].

By the second week of infection, a rapid expansion of latently infected cells is observed in the spleen [46], which is thought to be the major latency reservoir during persistent infection. Latency in the spleen is initially established in three different cell types, B-lymphocytes, macrophages and DCs [47]. While B-cells represent the majority of latently infected cells, they are not essential for the establishment of latency as other cell types can sustain persistent infection in B-cell deficient animals [41]. Around two weeks post infection, the frequency of infected splenocytes reaches a peak and the majority of infected cells have the phenotype of activated germinal center B-cells [48].

Concomitantly with the establishment of latency in the spleen, a marked splenomegaly develops in infected mice in the first 2-3 weeks post infection (Fig. 3).

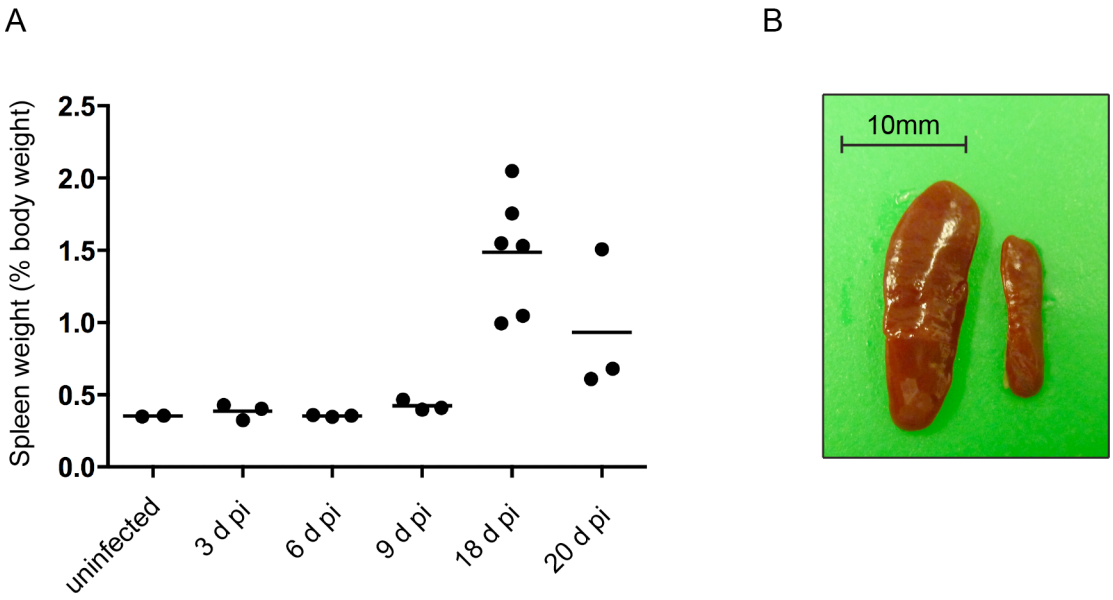


Figure 3. Splenomegaly in C57BL/6 mice after MHV-68 infection. A) Spleen weight relative to total body weight transiently increases after infection (d pi = days post infection). B) Enlarged spleen from a mouse on day 18 post infection (left) in comparison to a spleen from an uninfected mouse (right).

MHV-68-induced splenomegaly is caused by an increase in the numbers of B-cells, $CD4^+$ and $CD8^+$ T-cells, but most pronounced is the increase in $CD8^+$ T-cells [49]. The phenomenon is dependent on the presence of B-cells as well as $CD4^+$ T-cells, depletion of which has been shown to result in absence of splenomegaly [45]. While splenomegaly generally resolves around 1 month post infection, a pronounced lymphocytosis prevails in the peripheral blood of infected mice for at least another month [50], which bears striking

resemblance to the Infectious Mononucleosis (IM) syndrome associated with primary EBV infection [51]. Similar to IM patients, where mono- or oligoclonal populations of $CD8^+$ T-cells make up the major part of lymphocytosis in the blood [52], in MHV-68 infected mice, the numbers of activated $CD8^+$ T-cells in the periphery is elevated up to 4-fold. Interestingly, these T-cells show a significant skewing in T-cell receptor usage with up to 75% of $CD8^+$ T-cells expressing Vβ4 chain in their αβTCR [50]. The Vβ4-specific T-cell stimulatory activity does not seem to depend on classical MHC class I or II antigen presentation [53] and displays some features reminiscent of a viral superantigen [54]. Indeed, the gene product of MHV-68-specific ORF M1 was shown to be a secreted protein that has the ability to stimulate $V\beta4^+$ $CD8^+$ T-cells and act as immunoregulatory factor with superantigen-like properties [55].

As infection progresses, the pool of latently infected splenocytes decreases significantly, from about 1 in 100 cells to 1 in 20'000 cells by 3 months post infection. During this period, the fraction of non-B-cells and naive B-cells contributing to the latently infected cell pool diminishes greatly. At late time points post infection, latency is maintained at steady levels, presumably balanced by continuous immune surveillance and MHV-68 is found preferentially in surface IgD^- splenic B-lymphocytes that have the phenotype of long-lived memory B-cells [56]. The establishment of long-term latency by accessing the memory B-cell pool is a feature MHV-68 shares with the human virus EBV, which is found in the peripheral blood of healthy carriers exclusively in resting memory B-cells [57], and likely represents a conserved strategy of gamma-herpesviruses.

The maintenance of a steady level of infected cells indicates equilibrium between latency, reactivation and immune control that ensures life-long infection without adverse effects for the host. Notably, dose and route of inoculation do not influence the level of latently infected cells, as infections with a range of 6 logs of infectious MHV-68 particles injected intraperitoneally or a range of 5 logs of infectious particles administered intranasally resulted in comparable levels of infected splenocytes during long-term latency [58]. These results argue that a maximal level of latency is established in the spleen and that there are homeostatic mechanisms in place to ensure long-term infection in immunocompetent hosts.

Immune response to MHV-68

One of the biggest draws of the MHV-68 mouse model is the ability to study immunity-related host-pathogen interactions *in vivo*. The availability of a wide array of genetically engineered mouse strains that lack specific components of the immune system allows researchers to identify immune responses against different stages of gamma-herpesvirus

infection. Gamma-herpesviruses are difficult targets for the immune system since they are exquisitely adapted by co-evolution to establish life-long infections and avoid clearance in an immunocompetent host. As discussed above, infection with MHV-68 is characterized by distinct phases from acute infection in the lung, latency establishment in lymphoid organs with concomitant expansion of latently infected cells, subsequent contraction of the pool of infected cells and onset of long-term latency in memory B-cells. Consequently, different immune effector mechanisms are involved in controlling the distinct phases of MHV-68 infection, and immune responses that are effective against any particular phase of infection might be non-effective against another. Additionally, MHV-68 has evolved an array of measures to evade the immune responses and there is evidence that the virus even diverts cellular signaling pathways involved in immunity to drive certain aspects of its life cycle (discussed below).

Innate immunity

Interferon

In mammals, interferons (IFNs) are cytokines that constitute an important first line of defense against viral infection, hindering replication and therefore viral spread until adaptive immunity has had time to be mounted [59].

Mice deficient for the type I IFN receptor (*ifnr*$^{-/-}$) undergo an aggressive infection of the lung upon *i.n.* inoculation, with virus titers 100-1'000 fold higher than those in congenic wild-type mice [60]. Consequently, up to 90% of these mice succumb to high doses of virus due to interstitial pneumonia with focal hemorrhage and necrosis and still 30% of mice do not survive low-dose infection, indicating that IFN I signaling plays an important role in limiting initial acute replication. *ifnr*$^{-/-}$ mice also show 10-fold higher numbers of latently infected cells in the spleen initially, but 3 weeks after infection, frequencies of latently infected splenocytes are similar in *ifnr*$^{-/-}$ and wt mice, indicating that type I IFNs are dispensable for controlling latency. Type I IFNs might still play role in regulating viral latency, since infected splenocytes lacking type I IFN receptor were found to reactivate with higher efficiency *ex vivo* than splenocytes from wt mice [61]. How this observation translates to the situation *in vivo* is not entirely clear.

In contrast to type I IFNs, type II IFN does not appear to be required for clearance of acute infection in the lung. Mice deficient for IFN-gamma receptor (*ifngr*$^{-/-}$) showed no difference in viral titers or clearance of MHV-68 from the lung [62,63]. Despite the apparently unimpaired control of lytic infection, *ifngr*$^{-/-}$ mice succumb later during infection to

severe incidents of inflammatory pathologies such as large vessel arteritis [64] or multi-organ fibrosis [65]. At 5-12 months post infection, *ifngr*$^{-/-}$ mice can develop inflammatory lesions in the lungs that progress to lymphoid hyperplasia or pulmonary lymphoma [66]. These observations indicate that mice deficient in type II IFN are impaired in controlling chronic infection rather than the acute phase in the lungs. Indeed, IFN-gamma was shown to suppress reactivation of MHV-68 from latently infected cells [67], suggesting that the inflammatory pathologies observed in *ifngr*$^{-/-}$ mice might be due to persistent reactivation and replication of virus in lung tissue. An important caveat when discussing the role of IFN-gamma in the host response to MHV-68 is the observation that dependence on type II IFN signaling for successful clearance of acute infection from the lung is dependent on the mouse strain. While *ifngr*$^{-/-}$ mice on a C57BL/6 genetic background are able to control pulmonary infection as efficiently as wild-type controls, *ifngr*$^{-/-}$ on a BALB/c background are impaired in clearance of MHV-68 from the lung as evidenced by higher viral titers and increased mortality [68].

Toll-like receptor signaling

The family of Toll-like receptors (TLRs) is an integral part of innate immunity whose function is to recognize non-host structures and molecular patterns, initiate an inflammatory response through expression of cytokines, and help to shape the adaptive immune response [69]. Among the twelve TLRs described in mice so far [70], TLR7 and TLR9 recognize foreign nucleic acid and have been shown to be important in the recognition of viral infections. Upon binding of their respective ligand, TLR7 and TLR9 signal via a shared pathway through the adaptor molecule myeloid differentiation primary response protein 88 (MyD88) that culminates in the activation of the transcription factor nuclear factor κB (NFκB), which induces expression of cytokines, adhesion molecules and other cellular mediators that serve to activate and prime the adaptive immune response.

Mice that are deficient for the TLR-adaptor protein MyD88 and therefore lack TLR7- and TLR9-mediated signaling clear acute infection in the lungs without notable differences to the wt controls, indicating that TLR7 or TLR9 signaling is not crucial for clearance of MHV-68 from the lungs. Latency establishment in the spleen was significantly disturbed though, resulting in a 10-fold decrease in the frequency of infected cells. Infection of wt x *myd88*$^{-/-}$ bone-marrow chimeric mice confirmed that MHV-68 efficiently established latency in *myd88*$^{+/+}$ B-cells but not in *myd88*$^{-/-}$ B-cells [71]. The observed impairment of MHV-68 to establish latency in cells without functional MyD88 argues for an important role of TLR7 and TLR9 signaling in latent infection and illustrates the hijacking of a cellular immune pathway

by MHV-68 for its own benefit. This hypothesis is further substantiated by the observation that a recombinant strain of MHV-68 that suppresses NFκB activation in infected cells by constantly expressing the inhibitory molecule IκBα, had a similar phenotype *in vivo* with substantially decreased numbers of latently infected cells in the spleen [72]. Besides supporting latent infection, NFκB activation also exhibits protective properties however, since mice deficient for NFκB subunit p50 have 10- to 100-fold higher viral titers in the lungs early after i.n. inoculation. Despite this apparent deficiency in limiting acute replication, *p50*$^{-/-}$ mice are nonetheless able to clear acute infection [73].

Taken together, published data suggest that gamma-herpesviruses have evolved mechanisms to usurp important signaling pathways of the innate immune system to ensure long-term persistence in infected cells and possibly also to regulate viral gene expression and thus their life-cycle.

Natural killer (NK) cells

The cellular component of the innate immune system consists of natural killer (NK) cells that have the ability to induce apoptosis in cells displaying altered expression of surface molecules such as virus-infected cells or cancer cells. NK cells have been shown to be important in the immune response to alpha-herpesvirus HSV-1 [74] and beta-herpesvirus cytomegalovirus [75]. In contrast, NK-cell depletion or genetic deficiency of NK cells did not alter the course of lytic or latent MHV-68 infection in mice [76]. NK cell populations in the mouse are found to be elevated shortly after MHV-68 infection and these cells show markers of activation and prove capable of cytotoxic killing of target cells *in vitro* upon explant, however, depletion of NK cells does not impact the viral titers in the lungs [77]. Thus, the contribution of NK cells to the control of MHV-68 seems negligible. This contrasts the observations related to EBV-associated B lymphocyte transformation [78] (Lünemann A et al. JI 2013, in press).

Adaptive immunity

CD8$^+$ T-cells

CD8$^+$ cytotoxic lymphocytes (CTLs) play a major role in controlling primary lytic MHV-68 infection. While viral titers in the lungs normally peak around 3 days post infection and infectious virus is cleared from the lungs approximately 1 week post infection, depletion of CD8$^+$ T-cells not only results in higher peak titers but also failure to clear infectious virus and thus increased pathology [79]. Similarly, *β2-microglobulin*$^{-/-}$ mice that lack CD8$^+$ T-cells were found to be unable to clear infectious virus from the spleen after intraperitoneal inoculation

[41], confirming the importance of $CD8^+$ T-cells in eliminating acute replication. In turn, mice that were primed with a recombinant vaccinia virus expressing the most prominent $CD8^+$ T-cell epitope of MHV-68 lytic cycle protein p56 have a strongly reduced productive infection in the lungs and initially delayed kinetics in the establishment of latency in the spleen. Eventually, latent infection in the spleen reaches levels of wt mice though, indicating that a primed $CD8^+$ T-cell response is very efficient at terminating acute replication but is not sufficient to prevent latency [80].

In addition to the crucial role in the control of acute replication in both the lungs and spleen, $CD8^+$ T-cells apparently also regulate latency at later time points, as *cd8*$^{-/-}$ mice were found to have about 6-fold higher frequencies of latently infected splenocytes [81]. The $CD8^+$ T-cell response developing after inoculation of MHV-68 is directed against a broad repertoire of epitopes, identified from early and late lytic gene products [82]. Interestingly, the $CD8^+$ T-cell response seems to occur in two distinct phases that are dominated by CTL specific for two different gene products, ORF6 (p56) and ORF61 (p79). $CD8^+$ T-cells specific for p56 epitope dominate the early phase of infection during the peak of lytic replication in the lung but decline rapidly afterwards while responses against p79 emerge slower, peaking during latency establishment but are sustained longer, especially in the spleen [83]. The difference in kinetics between the two populations, together with the observation that p56-specific effector cells efficiently lysed virus-infected fibroblasts *in vitro* while p79-specific cells were less effective, suggest that the $CD8^+$ T-cell response is mounted in two waves, one targeting replication in epithelium and another one targeting replication in B-cells during latency establishment.

Notably, the $CD8^+$ $V\beta4^+$ T-cell population dominating during the infectious mononucleosis-like syndrome does not show specificity for any viral epitope tested so far and emerges later than the specific $CD8^+$ T-cell population. A contribution of these cells to the immune response against MHV-68 therefore seems unlikely. Rather, the expansion of $CD8^+$ $V\beta4^+$ cells through a MHC class I-independent mechanism like a viral superantigen as has been speculated (see above), potentially serves to divert the immune system long enough to successfully establish long-term latency.

$CD8^+$ T-cells also play a role in controlling latent infection, but the viral epitopes involved in recognition of latently infected cells are not very well known so far. The only latency associated viral gene product shown to elicit specific $CD8^+$ T-cell populations is M2 [84]. M2 has no known homologues in other gamma-herpesviruses and is only transiently expressed during establishment of latency 1-3 weeks after *i.n.* inoculation. A population of

$CD8^+$ T-cells specific for M2 was observed 2-3 weeks post infection, coincident with the decline in numbers of latently infected cells in the spleen. Consistent with the idea of this subset of $CD8^+$ T-cells being instrumental in controlling the number of latently infected cells, adoptive transfer of an M2-specific T-cell line led to 30-80-fold reduction of infected splenocytes at day 14 post infection without apparent differences in lung viral titers. Vaccination with the M2 epitope resulted in a transient reduction of latently infected splenocytes but this did not translate into reduced long-term latency, indicating that infected splenocytes might only be susceptible to $CD8^+$ T-cell attack in a limited time window during latency establishment but not during long-term latency [85].

Cytotoxicity mediated by specific $CD8^+$ T-cells appears to be one of the most important immune responses to MHV-68 infection. Cytotoxic lymphocytes seem to be rather successful in fighting productively replicating virus both during early infection in the lung as well as during latency establishment in the lymphoid compartment. However, $CD8^+$ T-cell responses are evidently unable to efficiently eliminate latent virus and $CD8^+$ T-cell evasion is likely a driving force behind the evolution of gamma-herpesvirus latency with minimal viral gene expression.

$CD4^+$ T-cells

The role of $CD4^+$ T-cells is much less understood than of $CD8^+$ T-cells. Recent studies underscore an important contribution of $CD4^+$ T-cells to controlling gamma-herpesviruses and indeed, $CD4^+$ T-cells specific for MHV-68 are detected early after infection and appear to retain an activated phenotype for at least 1 year [86].

Antibody-mediated depletion of $CD4^+$ T-cells causes a slight delay in peak viral titers in the lungs after *i.n.* inoculation, but the mice are still able to clear acute infection, albeit with slower kinetics [79]. Interestingly, the proliferation of splenocytes at the onset of latency leading to the characteristic splenomegaly and latency amplification seen in immunocompetent mice is absent in CD4-depleted mice. Consequently, CD4-depleted mice show lower frequencies of infected cells in an infectious center assay compared to controls. Similar results were obtained after *i.p.* infection of CD4-depleted mice, splenomegaly was absent and the number of infectious centers dropped five-fold. However, from day 30 post infection onwards, the frequencies of infected cells in depleted and non-depleted mice were similar [49]. These experiments provide evidence that $CD4^+$ T-cells are not necessary for MHV-68 clearance in the lungs but instrumental in driving lymphoproliferation in the spleen leading to splenomegaly and infectious mononucleosis-like syndrome. They might actually

have a beneficial function for MHV-68 during latency establishment in the first 2-3 weeks post infection by increasing the number of infected cells. Nevertheless, MHV-68 was able to establish long-term latency at a similar level with or without splenomegaly, so the initial expansion of the latent reservoir does not appear to be vital for long-term infection. While the $CD4^+$ subset of T-cells has no influence on long-term latency, it clearly plays a role in the control of acute infection as evidenced by the fact that depletion of either $CD4^+$ or $CD8^+$ cells leads to a slight delay in clearance but not complete failure, while double depletion of $CD4^+$ and $CD8^+$ cells results in loss of control over acute infection and death [87].

The question arises whether $CD4^+$ T-cells have direct effector function or they contribute to control of MHV-68 infection by providing help to other effector mechanisms either directly or through cytokine expression. The data collected up to date indicate that it is probably a combination of both. There is evidence for a role of cognate $CD4^+$ T-cell help in maintaining long-term control of MHV-68 reactivation by $CD8^+$ T-cells, since in mice deficient in MHC class II (*mhcII*$^{-/-}$), progressively rising titers of infectious virus are found in the respiratory tract that are ultimately fatal [88].

An intrinsic, helper-independent role of $CD4^+$ T-cells is suggested by the finding that ovalbumin-specific $CD4^+$ T-cells are able to limit replication of a recombinant, OVA-expressing MHV-68 strain and prolong survival of *rag*$^{-/-}$ mice in the absence of both $CD8^+$ T-cells as well as B-cells [89]. More recent studies documented direct effector functions of $CD4^+$ T-cells against MHV-68. Purified $CD4^+$ T-cells isolated from persistently infected mice have been shown to be able to directly recognize and kill MHV-68 loaded cells in an *in vitro* cytotoxicity assay in a MHC-dependent fashion. Additionally, specific killing of MHV-68 loaded cells was observed in an *in vivo* cytotoxicity assay in persistently infected mice, which was abrogated upon depletion of CD4, indicating the existence of MHV-68-specific lysis mediated by $CD4^+$ T-cells [90]. The dual role of $CD4^+$ T-cells as a cytolytic effector and as a helper is further consolidated by the identification of two distinct $CD4^+$ T-cell populations emerging in MHV-68 infected mice. One of the populations consists of steady state IFNg producers that are independent of antigenic restimulation *in vitro*, in contrast to virus-specific $CD8^+$ T-cells that require antigenic stimulation for IFNg production. A second population stained positive for the surrogate marker of cytolytic T-cells, CD107, but not for intracellular IFNg, indicating that separate populations of $CD4^+$ T-cells mediate cytolytic activity and cytokine production [91].

Antibody response

As discussed above, the cellular components of the adaptive immune response play important roles in fighting MHV-68 infection, especially in controlling initial acute infection. The antibody response is likely too slow to develop to substantially contribute to these early events. Significant virus-specific IgG titers appear only after day 10 post infection, at a time when acute infection in the lungs has already been resolved, and these titers continue to increase progressively until day 70 after infection [92]. Total IgG levels in the serum strongly increase from day 10 after infection, most likely due to the activation and proliferation of splenic B-cells during splenomegaly, but the fraction of IgG specific for MHV-68 is small. Loss-of-function studies to investigate the importance of a functional antibody immune response are somewhat limited since B-cells are the major target for latent infection. While MHV-68 manages to establish latent infection in B-cell-deficient mice, it is unclear how representative this kind of long-term infection is. Nevertheless, adoptive transfer of polyclonal antibody serum against MHV-68 can reduce the frequency of infected splenocytes in B-cell-deficient mice, indicating that antibodies might play a role in controlling latent infection, at least in non-B-cells [93]. The efficacy of antibody-related immunity in immunocompetent hosts is questionable, however, since MHV-68 is able to successfully establish latency despite high titers of virus-specific antibodies at later times of infection. Notably, while immune sera successfully blocked MHV-68 infection of fibroblasts, infection of Fc receptor-positive macrophages and dendritic cells was increased by neutralizing antibodies, arguing that Fc receptor-mediated uptake might present an alternative route of infection in susceptible cell types [94].

Immune evasion by MHV-68

Gamma-herpesviruses have evolved a life cycle that relies on long-term infection of the host with sporadic reactivation to ensure transmission. In order to enable long-term latency, MHV-68 has developed several strategies common among gamma-herpesviruses and some specific to MHV-68 to overcome i) host immunity and ii) apoptosis of infected cells.

As discussed in the above chapter, a variety of immune mechanisms contribute to the control of MHV-68 infection, but cytotoxic activity of $CD8^+$ T-cells seems to be one of the most efficient. To counteract $CD8^+$ T-cell activity is especially important during lytic infection when many viral genes are expressed and antigens are abundant.

MHV-68 interferes with $CD8^+$ T-cell activation by inhibiting MHC class I-dependent antigen presentation. Mouse embryonic fibroblasts expressing a specific CTL epitope are

protected from lysis after infection with MHV-68. Detailed analysis of the MEFs revealed that the expression of MHC class I proteins on the surface was strongly reduced after MHV-68 infection and genetic analysis identified viral protein MK3 as the mediator of MHC-I suppression [95]. MHV-68 encoded protein MK3 locates to the endoplasmatic reticulum where it binds H-2D chains via a zinc-finger binding motif and targets them for proteasomal degradation [96]. *In vivo*, MK3 is expressed both during lytic replication as well as during latency establishment in the spleen. A recombinant virus deficient for MK3 was cleared from the lungs with similar kinetics as a wt strain, however, the number of latently infected splenocytes was reduced while the frequency of MHV-68-specific CTLs was increased, indicating that interference with MHC class I-dependent antigen presentation is important during latency expansion in the spleen [97].

Downregulation of MHC class I expression on the surface of infected cells in order to evade T-cell recognition is also found in other members of the gamma-2-herpesvirus family, albeit through slightly different mechanisms. KSHV expresses two proteins, K3 and K5, that reduce MHC class I on the surface of infected cells by triggering rapid endocytosis of HLA chains [98,99].

Another strategy to evade host immune response, which is employed by many viruses, is interference with chemokine signaling. Chemokines regulate the recruitment of leukocytes to the site of inflammation, and herpesviruses have been shown to intervene in this process by expression of chemokine homologs (KSHV, hCMV, mCMV) and chemokine receptor homologs (KSHV, HVS, MHV-68, CMV) [100]. MHV-68 has an additional mechanism that is so far not known of any other herpesvirus. The gene M3 encodes a 44-kD secreted protein that exhibits broad-spectrum chemokine-binding properties, a feature also described in poxviruses. M3 binds to members of all chemokine subfamilies and blocks interaction with high-affinity receptors, preventing intracellular chemokine signaling and calcium influx [101,102]. It is expressed during acute infection and early latency [103] and is necessary for the establishment of normal latent viral load. Disruption of M3 does not change viral titers or the course of acute infection in the respiratory tract, but virus-driven activation of B-cells typical for the infectious mononucleosis-like syndrome is greatly reduced in the spleen and consequently there are far fewer cells positive for viral-tRNA. Depletion of $CD8^+$ T-cells restored the levels of splenocytes infected with the M3-deficient virus to levels of the wild-type strain, suggesting that the main role of M3 during latency amplification is to protect infected cells from CTLs [104]. Even though M3 is mainly expressed at early times post infection and not long-term latency, it might also be implicated in MHV-68-associated

lymphomagenesis as it has been shown to efficiently protect tumor cells from CTL attack. Supernatant of tumor cells transfected with M3 are able to inhibit migration of tumor-specific CTL *in vitro*, and secretion of M3 protects transfected tumor cells and surrounding cells from otherwise effective tumor-specific CTLs *in vivo* [105].

Besides the described $CD8^+$ T-cell evasion, gamma-herpesviruses have a tendency to interfere with intracellular IFN type I signaling. Type I IFNs do not only promote inflammation by activating lymphocytes, NK-cells and macrophages but also have auto-and paracrine functions that induce antiviral effects in infected cells such as inhibition of RNA expression and protein synthesis, degradation of mRNA and induction of apoptosis. To counteract IFN type I signaling, MHV-68 expresses an enzyme (ORF54) which induces degradation of the type I interferon receptor IFNAR [106]. Experimental infection of mice with a recombinant virus deficient for ORF54 revealed only slightly elevated viral titers in the lung but a substantial reduction in splenic infection 14 days post inoculation, underscoring the importance of blocking type I IFN signaling in order to establish latency. To further disrupt IFN function, MHV-68 encodes a kinase (ORF36) that inhibits IFN expression by binding to IRF3, a transcription factor that activates IFN promoters [107]. Homologs of ORF36 are found in all members of the herpesvirus family [108] and their ability to suppress IRF3 function is conserved in all gamma-herpesviruses [109,110], illustrating the evolutional pressure to disturb IFN function.

Apoptosis is a highly efficient defense mechanism to limit viral replication and persistence that herpesviruses must overcome in order to establish long-term infection. Consequently, all lymphotropic herpesviruses have evolved strategies to inhibit induction of apoptosis in the host cell [111]. Apoptosis can be triggered not only by cytolytic effector cells but also through cell intrinsic pathways in response to metabolic stress or DNA damage which can be induced by excessive replication of intracellular pathogens.

The balance between pro- and anti-apoptotic members of the bcl-2 protein family regulates the cell intrinsic pathways leading to apoptosis and homologues to bcl-2-family proteins that encode characteristic BH1 and BH2 domains have been found in all gamma-herpesviruses. In contrast to cellular bcl-2 proteins that can be converted to potent pro-apoptotic molecules through caspase-mediated cleavage, herpesviral bcl-2 homologues cannot be cleaved by caspases and thus retain their anti-apoptotic potential [112].
EBV gene product BHRF1 colocalizes with bcl-2 in vitro and protects cells from apoptosis [113], ORF16 from HVS and KSHV are v-bcl-2 proteins that dimerize with bcl-2 to suppress apoptosis [114,115] and in MHV-68, a putative viral bcl-2 was identified in M11. Even

though homology to bcl-2 and EBV BHRF1 is limited, functionality of the protein is conserved as M11 was shown to inhibit apoptosis induced by anti-Fas antibody or tumor necrosis factor α (TNF-α) when transfected into HeLa cells [116]. M11 is expressed together with early transcripts during lytic infection of fibroblasts *in vitro*, probably playing a role in survival of productively infected cells to ensure maximal viral replication. *In vivo*, M11 transcripts are abundant in lung and spleen tissue at 2 weeks post infection together with other lytic gene products such as ORF50 and gp150, reflecting expression during lytic replication [117]. Interestingly, M11 transcripts are also found 10 months post infection in the spleen and lungs of infected mice, while other lytic transcripts are absent, indicating that M11 is also expressed during long-term latency where it might serve to protect infected cells from Fas-mediated lysis by cytotoxic immune cells. Due to their ability to inhibit programmed cell death, herpesviral bcl-2 homologues might not only be important as an immune evasion mechanism for the establishment of life-long infection, but also play a major role in the ontology of gamma-herpesvirus-associated cancers.

MHV-68 and lymphomagenesis

One of the hopes put into the MHV-68 mouse model is to be able to study the oncogenic properties of gamma-herpesviruses and to understand how persistent infection is linked to lymphomagenesis in the host. However, there seem to be substantial differences in the oncogenic potential between the two subgroups of gamma-herpesviruses. Originally, it was assumed that only members of the subgroup *lymphocryptovirus*, to which EBV belongs, are able to growth transform primary B-cells *in vitro*, while B-lymphotropic *rhadinoviruses* MHV-68 and KSHV were not. Studies of the immortalization of B-cells by EBV identified a set of nine latency genes (EBNA1-6; LMP1; LMP2A; LMP2B) that in concert regulate the transformation to proliferating lymphoblastoid cell lines and were therefore termed the growth program [118]. In *rhadinoviruses*, there is no evidence for an analogous growth program and even though latency-associated genes have been identified in both KSHV and MHV-68, there are no homologues to the EBV latency genes. Still, KSHV is associated with several forms of cancer in humans [8,9] and experiments with T-lymphotropic *rhadinoviruses* Herpesvirus saimiri and Herpesvirus ateles showed growth-transformation of primary T-cells *in vitro*, proving that members of this subclass are very well oncogenic [119,120]. Indeed, a recent study has shown that MHV-68-infection of progenitor B-cells isolated from murine fetal liver results in proliferating plasmablasts that retain the virus and can be propagated indefinitely, thus reminiscent of EBV-transformed lymphoblastoid cell lines [121].

In vivo, chronic long-term infection with MHV-68 was reported to be associated with increased incidence of lymphoma. In BALB/c mice, 9% of infected animals developed lymphoproliferative disorders including high-grade lymphomas in lymph nodes, spleen, liver and kidney [122]. Histological characterization revealed a mixed composition of B- and T-cells in most cases and only very few cells stained positive for viral genome by in situ hybridization. The development of lymphoma seems rather slow, with the earliest tumors found 6 months after infection and most only after 2 years and more. Immunosuppressive treatment using cyclosporin A increased tumor frequency to 50% but did not accelerate the process of tumorigenesis. Afterwards, research mainly focused on lymphoma models utilizing mouse strains with variable genetic immunodeficiencies. Infection of BALB/c mice deficient for β2-microglobulin ($\beta 2m^{-/-}$) that lack a functional $CD8^+$ T-cell response increased the incidence of lymphoma to 67% in comparison to 22% in mock infected controls over a timeframe of about 8-9 months [123]. Consistent with earlier observation, only a small number of lymphoma cells stained positive for viral tRNAs, indicating that MHV-68-associated lymphomagenesis does not require constant presence of virus in the transformed cells but rather a "hit-and-run" type of transformation, in contrast to EBV-associated endemic Burkitt's Lymphoma where usually a vast majority of cancer cells carry viral genome. Notably, long-term infection of a mouse strain with different genetic background (129) bearing the same genetic deficiency ($\beta 2m^{-/-}$) did not result in any lymphoma over a timeframe of 1.5 years, confirming the impression that MHV-68 infection and pathology are strain-dependent (see above). In IFNg-receptor deficient C57BL/6 mice, MHV-68 infection was associated with high incidence of inflammatory lesions in the lungs that progressed to pulmonary lymphoma in 45% of mice, a pathology similar to Lymphomatoid granulomatosis, a rare EBV-associated lymphoproliferative disease in human patients [66].

Evidence from long-term infection *in vivo* suggests that lymphoproliferative diseases are not a likely outcome of MHV-68 infection in immunocompetent hosts, presumably because i) MHV-68 lacks latency genes to efficiently growth-transform its host cell, and ii) adaptive immune responses are efficient at eliminating cells that undergo virus-driven hyperproliferation. So far, the only MHV-68 gene to show oncogenic potential comparable to EBV latency genes is the viral cyclin D homologue (ORF72), which drives cell cycle progression when expressed in thymocytes and leads to lymphoma in ORF72-transgenic mice [124]. In contrast to the EBV latency genes however, ORF72 is expressed at late times during lytic infection of fibroblasts but was so far not found during latent infection [125].

Immune control of MHV-68 associated tumors was investigated using B-cells immortalized by MHV-68 *in vivo* or *in vitro* that are able to establish growing tumors in immune deficient nude mice. In BALB/c nude mice, adoptive transfer of $CD4^+$ T-cells led to a regression of tumors caused by injection of MHV-68 infected B-cell lymphoma S11, while adoptive transfer of $CD8^+$ T-cells and B-cells was not effective [126]. In contrast, both $CD4^+$ and $CD8^+$ T-cells were effective at controlling the outgrowth of B-cells immortalized by MHV-68 infection *in vitro* when transferred into *rag2*$^{-/-}$ C57BL/6 mice [127].

To test oncogenic properties of other strains of murid Herpesvirus 4, long-term infection experiments were conducted with MHV-72, a strain isolated at the same time as MHV-68. Formation of neoplasms was reported in BALB/c mice at a rate similar to MHV-68 (13% vs 9%) at equally late times post infection [128]. Consistent with the findings from MHV-68, the incidence of MHV72-associated lymphoma can be increased by immunosuppressive drugs [129].

Toll-like Receptors

Toll-like receptors in innate immunity

Innate immune mechanisms constitute the first line of defense against invading microorganisms, are crucial for mounting a rapid innate immune response and to shape the slower primary adaptive immune response. The innate immune system recognizes pathogens via an array of germline-encoded pattern-recognition receptors (PRRs) that are triggered by common molecular structures of bacterial, viral, fungal, or parasitic pathogens. PRRs initiate signaling pathways that lead to the expression of microbicidal molecules and pro-inflammatory cytokines.

The first family of PRRs to be described was the Toll-like receptor family (TLRs), named after the Toll protein of the fruitfly (*Drosophila melanogaster*). Originally described as an important factor in embryonal dorso-ventral patterning of *Drosophila* [130], it was found that the Toll protein is also essential in the protection from fungal infections [131], indicating its role in innate immunity. Toll-like receptors are an evolutionarily conserved mechanism present in all mammals and the first homologue to the Toll protein of *Drosophila* in humans was found soon after [132]. To date there have been 10 and 12 TLRs identified in humans and in mice, respectively, with TLR1-TLR9 being conserved in both. Ligands for all TLRs except TLR10 have been identified and each TLR recognizes certain pathogen-

associated molecular patterns (PAMPs) including lipids, lipoproteins and nucleic acids that are present in pathogens such as bacteria, viruses, fungi and protozoa.

TLRs are membrane-spanning receptors that consist of an ectodomain containing leucine-rich repeats that are responsible for recognition of the ligand, a transmembrane domain and a cytoplasmic tail containing Toll-Interleukin 1 (IL1) receptor (TIR) domains that allows binding of distinct adaptor molecules for signal transduction [133]. Signal transduction via different cellular kinases ultimately leads to the activation of transcription factors that regulate the expression of pro-inflammatory cytokines, anti-viral effector molecules such as type I Interferon and upregulation of co-stimulatory receptors on cells of the adaptive immune system [134].

TLRs can be broadly divided into two groups based on their location on either the plasma membrane or the endosomal compartment. TLRs 1, 2, 4, 5, 6, 10 are located on the plasma membrane where they recognize predominantly hydrophobic structures such as proteins, lipopeptides and polysaccharides, whereas TLRs 3, 7, 8, 9 are located almost exclusively in the endosome where they sense different forms of nucleic acid [135].

Toll-like receptors and gamma-herpesviruses

Several members of the TLR family have been shown to sense gamma-herpesvirus infection. Since nucleic acids are the most potent immune stimulatory component of viruses, the TLRs mainly involved in herpesvirus recognition are the endosomal TLRs 3, 7, and 9. TLR9 recognizes unmethylated CpG motifs that are relatively rare in vertebrate DNA but abundantly present in herpesviral genomes and senses members from all herpesvirus subgroups [136,137] including EBV [138]. The ligand of TLR3 is double-stranded RNA, a molecular pattern that is not inherently present in herpesvirus genomes but can be generated as intermediate structures during replication and act as TLR3 agonist [139]. In the case of EBV, latency-associated EBV-encoded small RNAs (EBERs) are an additional ligand for TLR3 [140]. TLR7 is responsive to single-stranded RNA and has been reported to complement TLR9 signaling to generate an efficient immune response against murine Cytomegalovirus even though the exact nature of the viral agonist is not known [141].

TLR7 and TLR9 share a common signaling pathway through recruitment of adaptor molecule myeloid differentiation primary response protein 88 (MyD88) that binds to the cytoplasmic tail of activated TLRs via a homologous TIR-domain. MyD88 activates IL1-receptor-associated kinases (IRAKs) and tumor-necrosis-receptor-associated-factor 6 (TRAF6) that activate Inhibitor of nuclear factor κB-kinase complex (IKK-complex) (Fig.4).

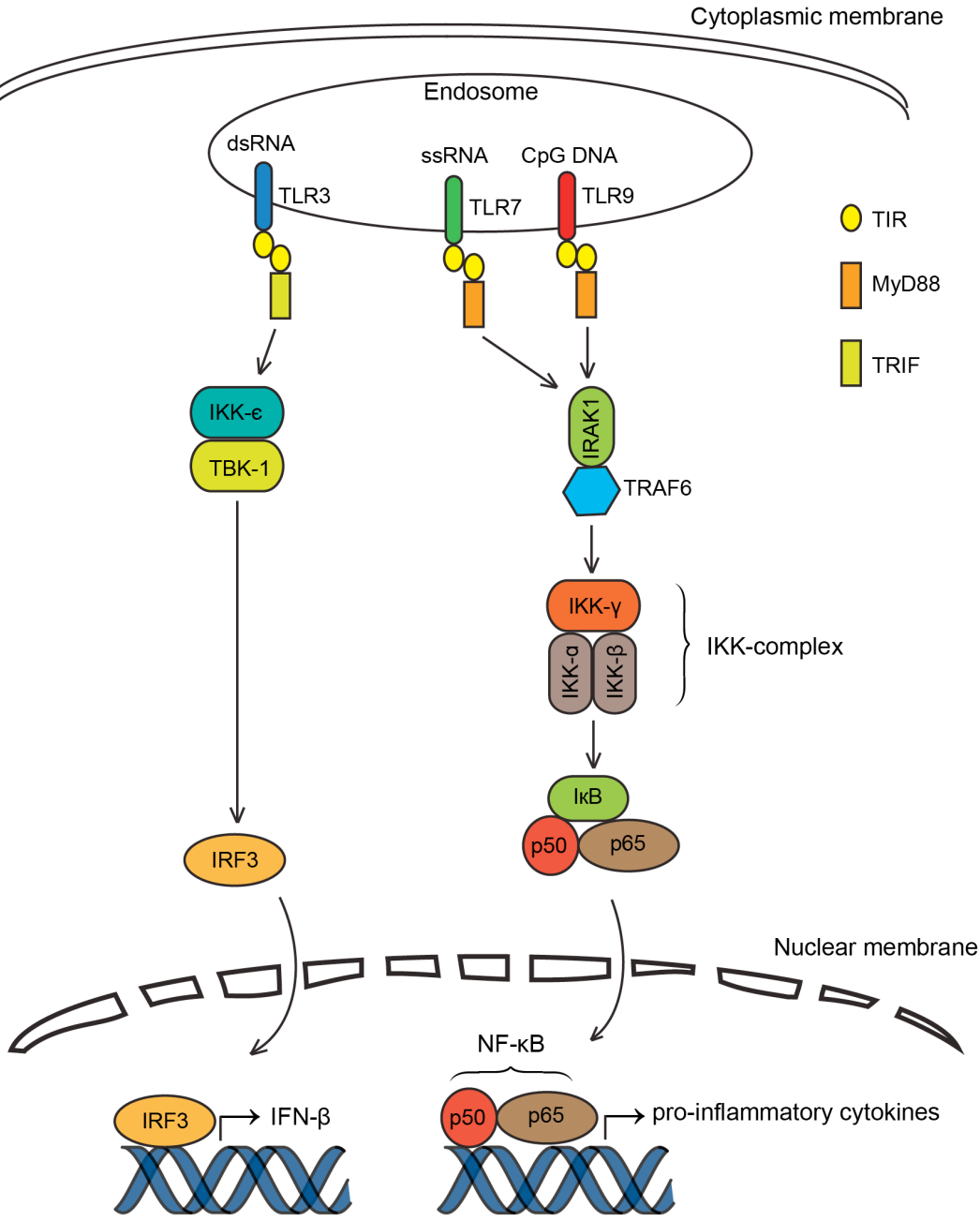


Figure 4. Signaling pathways of endosomal Toll-like receptors. TLR7 and TLR9 share a common signaling pathway through myeloid differentiation primary response protein 88 (MyD88), activation of IL1-receptor-associated kinase 1 (IRAK1) and tumor-necrosis-receptor-associated-factor 6 (TRAF6). Subsequent activation of Inhibitor of nuclear factor κB-kinase complex (IKK-complex) frees NFκB from its inhibitor IκB and allows nuclear translocation where expression of pro-inflammatory cytokines is induced. TLR3 signals via TIR-domain-containing adaptor molecule inducing IFN-β (TRIF), IKK-ε and TANK-binding kinase 1 (TBK1) to activate Interferon regulatory factor 3 (IRF3) which induces expression of IFN-β. Adapted from Akira & Takeda, Nat Rev Immunol. 2004 [142]

The transcription factor NFκB is released from its inhibitor in the cytoplasm and translocates to the nucleus where it induces expression of pro-inflammatory cytokines. TLR3

uses a different adaptor molecule, TIR-domain-containing adaptor molecule inducing IFN-β (TRIF), to relay its signal to IKK-ε and TANK-binding kinase 1 (TBK1) which activate Interferon regulatory factor 3 (IRF3) and induce expression of IFN- β (reviewed in [142]).

Signaling via endosomal TLRs and activation of NFκB transcription factors in the context of gamma-herpesvirus infection is not only important due to its role in the induction of host immune responses. There is evidence that TLR signaling contributes to shaping viral gene expression and thus the balance between lytic replication and latency. Earlier studies in our lab found that stimulation of Burkitt's lymphoma cells with synthetic ligands to TLR9 suppresses EBV reactivation *in vitro* [143,144] and transfection of NFκB subunit p65 was reported to inhibit expression of gamma-herpesvirus lytic genes by directly interacting with the promoter sequence of the master lytic regulator gene [145]. Conversely, synthetic ligands to TLRs 3 and 9 were reported to induce reactivation of both MHV-68 and KSHV *in vitro* [146,147], suggesting that TLR stimulation might play a role in viral replication. Additionally, activation of the NFκB axis has a range of effects on lymphocytes, controlling immune cell proliferation and survival by inducing expression of the growth factor interleukin 6 (IL6) and tumor-necrosis factor (TNF) [148].

The properties of TLR-mediated NFκB signaling are of particular interest in the context of gamma-herpesvirus infection, as they might turn out to be a double-edged sword: on the one hand they are crucial for the induction of an adaptive immune response, on the other hand they might facilitate establishment of long-term infection and lymphomagenesis through induction of proliferation and survival of infected B-lymphocytes. Thus, stimulation of TLR pathways offers a possible explanation as to how co-infections might disturb the balance between infection and immune control in asymptomatic long-term latency and contribute to the ontology of gamma-herpesvirus- associated lymphoma in immunocompetent patients.

Subject of Investigation

The subject of this thesis was to investigate the impact of recurrent innate immune stimulation via endosomal Toll-like receptors on gamma-herpesvirus infection and lymphomagenesis using MHV-68 as a model. To adress this subject, the following topics were investigated:

1. **Does long-term infection of MHV-68 in immune deficient mice lead to development of lymphoproliferative disease?**

Long-term infection with MHV-68 in mice has been associated with lymphoproliferative disorders similar to the human gamma-herpesviruses EBV and KSHV. Such a model would be valuable to investigate the ontology of gamma-herpesvirus-associated cancer. Since MHV-68 is not transforming *in vitro* and lymphomagenesis in immunocompetent mice is inefficient, we tested the outcome of long-term infection in mice with specific immune deficiencies.

2. **How does stimulation of endosomal Toll-like receptors impact on MHV-68 infection *in vitro* and *in vivo*?**

Toll-like receptor signaling affects host B-cells as well as viral gene expression. Studies in our lab have shown that TLR9 signaling suppresses EBV reactivation in Burkitt's lymphoma cells *in vitro*. We tested the outcome of constant TLR stimulation on MHV-68 *in vitro* and *in vivo* using synthetic ligands for endosomal TLRs 7 and 9.

3. **Does the compound hemozoin derived from the malaria parasite *Plasmodium falciparum* mimick the impact of TLR stimulation on MHV-68 gene expression?**

Endemic Burkitt's Lymphoma is associated with both EBV infection and holoendemic transmission of the parasite *Plasmodium falciparum*, the causative agent of Malaria tropica. Hemozoin is a waste product of *Plasmodium* metabolism in parasitized red blood cells and is constantly released into the bloodstream. Since hemozoin is a suspected ligand of TLR9, we hypothesized that *Plasmodium* infection impacts on gamma-herpesviruses by stimulating TLR pathways. We tested whether hemozoin mimicks the effect of synthetic TLR9 ligand on MHV-68 gene expression.

Results

MHV-68 latent infection is increased in mice with distinct immune deficiencies but lymphoproliferative disease is absent

Long-term MHV-68 infection is associated with lymphoproliferative disorders in mice and immune suppressive treatment has been reported to increase the incidence of malignancies. To study latent MHV-68 infection in immunocompromised mice, cohorts of C57BL/6 wild-type mice and congenic knock-out mice with distinct immune deficiencies were infected by intra-peritoneal injection. The knock-out mice used for this experiment were $CD8^{-/-}$, $CD4^{-/-}$ or $TLR9^{-/-}$. Three months post infection, mice were sacrificed and the frequency of infected splenocytes was measured by limiting-dilution nested PCR to assess establishment of latent infection (Fig. 5).

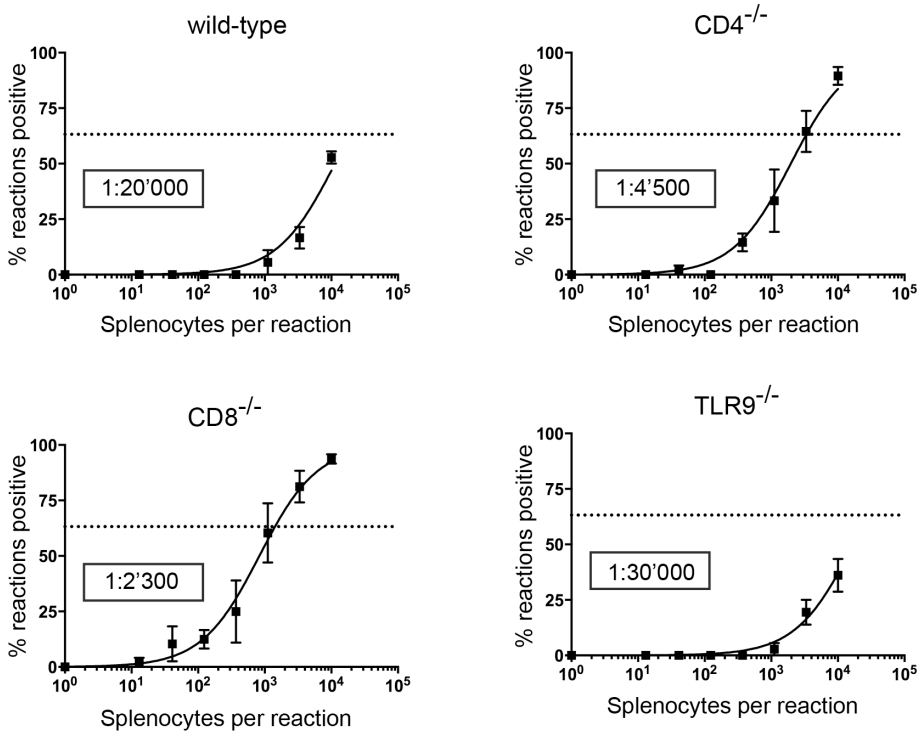


Figure 5. Intraperitoneal injection of MHV-68 leads to the establishment of latent infection in splenocytes *in vivo*. C57BL/6 wild-type and congenic knock-out mice (n=4 mice for each group) were infected with MHV-68 by i.p. injection. Splenocytes isolated from infected mice at 3 months post infection were analyzed by limiting-dilution nested PCR targeting ORF72. Data are expressed as mean percentages of positive reactions ±SD. Sigmoidal dose-response curve was fit by nonlinear regression analysis using GraphPad software. From the equation of the curve, the intersection with the dotted line at 63.2% was calculated and the frequency of MHV-68 positive splenocytes was assessed based on Poisson distribution. Calculated frequencies for each group are shown in the graph (number in the box). EC50 values of single curves were compared for statistical analysis using t-test (wt vs. $CD4^{-/-}$ p=0.005; wt vs. $CD8^{-/-}$ p=0.003; wt vs. $TLR9^{-/-}$ p=0.12).

In wild-type mice the frequency of infected splenocytes was about 1:20'000 cells. In mice with deficiencies in T-cell subsets ($CD4^{-/-}$, $CD8^{-/-}$), the frequency of splenocytes positive for viral genome was significantly elevated up to 10-fold, indicating a loss of immune control over latent infection. Nevertheless, none of the animals showed signs of clinical illness over the course of the experiment, suggesting that while a partial loss of immune control leads to higher levels of infection, redundant mechanisms protect the host from harmful disease. Mice lacking Toll-like receptor 9 showed a tendency to lower frequencies of infected cells, eventhough this difference was not significant. Notably, no infectious virus was detected in the spleens of any of the infected animals by plaque assay, indicating predominantly latent infection. After having confirmed successful establishment of latency in immunodeficient mice, infection was allowed to progress in two cohorts of infected $CD4^{-/-}$ and $CD8^{-/-}$ to investigate possible lymphoma formation. Health status of all animals was checked in weekly intervals. 11 months post infection, there was no sign of lymphoproliferative disorders and the experiment was terminated. All animals were sacrificed and analyzed post mortem for signs of lymphoma as well as limiting-dilution analysis of infected splenocytes (Fig. 6).

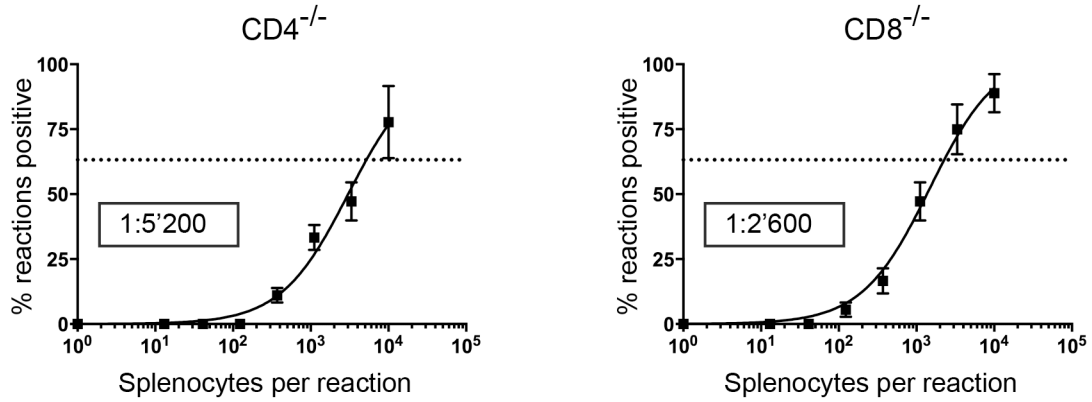


Figure 6. Frequencies of MHV-68 positive splenocytes in immune deficient mice are stable up to 11 months post infection. Knock-out mice on a C57BL/6 background (n=3 for $CD4^{-/-}$; n=4 for $CD8^{-/-}$) were infected with MHV-68 by i.p. injection. Splenocytes isolated from infected mice at 11 months post infection were analyzed by limiting-dilution nested PCR targeting ORF72. Data are expressed as mean percentages of positive reactions ±SD. Sigmoidal dose-response curve was fit by nonlinear regression analysis using GraphPad software. From the equation of the curve, the intersection with the dotted line at 63.2% was calculated and the frequency of MHV-68 positive splenocytes was assessed based on Poisson distribution. Calculated frequencies for each group are shown in the graph (number in the box).

Frequencies of infected cells at 11 months post infection did not differ significantly from the respective frequencies measured at 3 months post infection, suggesting that the level of latent infection is rather stable at least up to one year after initial infection. No signs of lymphoproliferative disorder were found post mortem.

Activation of NFκB via Endosomal Toll-like Receptors 7 or 9 Contributes to Limiting Murine Herpes Virus 68 Reactivation

Florian Haas, Kazuma Yamauchi, Monika Murat, Michele Bernasconi, Noboru Yamanaka, Roberto F. Speck, and David Nadal

manuscript submitted for publication

Abstract

Latent gamma-herpesvirus infection is associated with various B-cell lymphoma. In order to understand and possibly treat herpesvirus-associated malignancies, it is vital to understand the factors that control the balance between the two transcriptional states of gamma-herpesviruses, lytic replication and latency. In this study, we used murine gamma-herpesvirus (MHV)-68 as a model system to investigate how engagement of endosomal Toll-like receptors (TLR) impacts on reactivation *in vitro* and establishment of latent infection *in vivo*. We found that treatment with TLR7 ligand R848 and TLR9 ligand CpG ODN suppresses reactivation of MHV-68 *in vitro* and that this effect correlates with the ability to activate the transcription factor NFκB. *In vivo*, continuous stimulation of TLR7 by R848 treatment led to an increased frequency of infected splenocytes compared to mock-treated control. Downregulation of TLR9 by RNA interference *in vitro* reduced nuclear levels of NFκB p65 and consequently increased spontaneous reactivation in MHV-68 latently infected cells, indicating that the TLR9 pathway contributes to limiting spontaneous reactivation events. Frequencies of infected splenic B-cells in *tlr7*$^{-/-}$ or *tlr9*$^{-/-}$ mice did not differ from their wild-type counterpart. However, infected B-cells from *tlr9*$^{-/-}$ mice showed a higher frequency of reactivation compared to wild-type or *tlr7*$^{-/-}$ in an *ex vivo* reactivation assay. Our results show a suppressive effect of TLR7 and TLR9 signaling on MHV-68 reactivation and demonstrate that TLR9 signaling pathway is constantly active in infected cells, thereby contributing to NFκB activity and suppression of lytic replication.

for detailed information, see attached manuscript 1

Hemozoin Extracted from *Plasmodium falciparum* Cultures Suppresses Gamma-Herpesvirus Reactivation *in vitro*

Florian Haas, Michele Bernasconi, Hans-Peter Beck, Cristina Nevado, and David Nadal

manuscript in preparation

Abstract

Endemic Burkitt's lymphoma (eBL) is the most common pediatric cancer in equatorial Africa. It is associated with the gamma-herpesvirus Epstein-Barr virus and only occurs in regions where the malaria parasite *Plasmodium falciparum* is holoendemic. Thus eBL is suspected to be a polymicrobial disease. Although association with both EBV and malaria is established, the exact mechanism how chronic *P. falciparum* infection impacts on usually asymptomatic EBV and promotes incidence of EBV-positive cancer is unknown. Here, we show that hemozoin, a waste product of parasite metabolism extracted from *P. falciparum* cultures *in vitro,* suppresses lytic reactivation from latency in both EBV-infected BL cells as well as mouse lymphoma cells infected with murine gamma-herpesvirus 68. Suppression of viral reactivation was only seen when cells were stimulated with natural hemozoin extract but not with synthetic analogue β-hematin. Suppression of lytic reactivation promotes latency and has been shown to increase the frequency of infected cells *in vivo*. We hypothesize that chronic malaria contributes to the incidence of eBL by suppressing EBV reactivation via constant shedding of hemozoin to the bloodstream and therefore increasing the pool of latently infected B-cells.

for detailed information, see attached manuscript 2

Discussion and Outlook

MHV-68 infection of mice provides researchers a much-needed small and tractable animal model for gamma-herpesvirus infection, given that human gamma-herpesviruses can be studied *in vivo* only in non-human primates. The high degree of genetic homology among gamma-herpesviruses and the similarity in host cell subset tropism and viral life cycle makes MHV-68 a useful model system, despite minor concerns about infection in lab mice not properly reflecting the events in a natural host such as wood mice. The availability of genetically engineered mouse strains in conjunction with recombinant viruses enables uniquely detailed investigations of the importance of single cellular pathways or viral genes during different steps of host colonization. From what we know so far, it is reasonable to assume that events such as the resolution of acute infection and the immune control of latent MHV-68 are representative of gamma-herpesviruses in general and reflect universal concepts that are vital to understand in order to comprehend host-virus interaction. MHV-68 infection of mice therefore is an optimal tool to elucidate gamma-herpesvirus biology and host immune responses.

The long-term infection studies we performed using knock-out mice of different immune elements provide an example how the model can be used to investigate establishment of latency and control of persistent infection under very specific, well-defined conditions of immune deficiency. Our results illustrate how distinct arms of the immune system control gamma-herpesvirus infection. Even though infection levels were increased in immune deficient mice, no pathological signs of illness were observed, suggesting that redundant mechanisms of immune surveillance clear acute infection and control latent virus. We found the highest frequencies of infected splenocytes in mice lacking cytotoxic $CD8^+$ T-cells, a subset of immune cells that is important in controlling both acute and latent infection [81]. Mice lacking $CD4^+$ T-cells also showed a phenotype with higher levels of infected splenocytes, even though not as pronounced as $CD8^{-/-}$ mice. Interestingly, levels of latently infected splenocytes were rather constant over the timespan of the experiment, indicating that a balance was established between MHV-68 infection and immune control where various immune mechanisms contribute to maintaining latency at distinct levels.

Despite increased levels of latent MHV-68 infection in immune deficient mice, lymphoma formation was not observed until 11 months post infection. Therefore, although our sample size was small, the use of MHV-68 seems not to be apt to study pathogenic mechanisms of gamma-herpesvirus-associated lymphoma. Lymphoproliferative disorders

following MHV-68 infection seem to be very rare and slow to develop [122], rendering the respective studies rather cumbersome and unreliable. In β2-microglobulin$^{-/-}$ BALB/c mice that lack a functional CD8^{+} T-cell response, the incidence of lymphoma was increased to 67% after only 9 months of infection [123], an observation we could not confirm in CD8$^{-/-}$ mice from C57BL/6 background. It is worth noting that all MHV-68-associated lymphoma in immunocompetent mice reported so far have been observed in BALB/c mice, which indicates a substantial contribution of the genetic background to the susceptibility to lymphomagenesis. This makes general conclusions about the pathogenesis of MHV-68 difficult and the mouse genetic background should be considered in future experimental approaches.

MHV-68 lacks both the transforming potential of EBV *in vitro* as well as a distinct latency program of gene expression that defines various lymphoproliferative disorders caused by EBV in humans. For these reasons, it might be better to rely on other models for the study of EBV-associated lymphomagenesis such as reconstitution of a human immune system in immunodeficient mice and subsequent infection with EBV as described by Strowig et al. [149] or to use transgenic mice to model BL-like cancers through expression of the myc oncogene and EBV latent gene products like LMP2A [150,151].

Signaling via Toll-like receptors impacts on gamma-herpesvirus lytic gene expression and reactivation and it is therefore important to examine the associated signaling pathways *in vivo*. As shown by earlier studies in our lab using BL cells as a model [143], triggering of TLR9 invariably suppresses EBV reactivation. Using the MHV-68-infected cell line S11, we were able to confirm this interaction *in vitro*, indicating that it is not restricted to EBV but it is rather a general mechanism shared by gamma-herpesviruses. In contrast to the BL cell model, we unprecedentedly found that triggering of TLR7 also has the capacity to suppress spontaneous as well as induced reactivation (see manuscript 1). It is not surprising that TLR7 and TLR9 would have a comparable effect when stimulated, since they share a signaling pathway via the common adaptor molecule MyD88, which culminates in the activation of the transcription factor NFκB. Indeed, the suppressive effect on MHV-68 lytic genes correlated with the ability to induce NFκB activation, while TLR9-mediated suppression of EBV was independent of NFκB activation and was instead mediated by histone modifications on EBV-associated chromatin. It is interesting that two gamma-herpesviruses that diverged in their evolution about 60 million years ago retained the same feature but execute it via different mechanisms. This would imply that suppression of lytic genes via TLR stimulation is an important aspect of gamma-herpesvirus biology and confers significant advantages in biological fitness.

After having confirmed the suppression of lytic MHV-68 by endosomal TLR signaling inducing NFκB activation, we were able to study the consequences of repeated TLR stimulation *in vivo*. The treatment of infected mice with synthetic TLR7 ligand caused a significant increase of latently infected cells, suggesting that innate immune stimulation via TLR signaling is indeed reinforcing latency establishment rather than having an anti-viral effect for the host. Based on our observations *in vitro*, we hypothesize that activation of endosomal TLR pathways promotes establishment of latent infection by limiting initiation of lytic replication while concurrently enabling proliferation and survival of latently infected B-cells [152]. Activation of the transcription factor NFκB does not only seem to be beneficial but rather a prerequisite for MHV-68 latency, since a recombinant MHV-68 that expresses IκBαM, a constitutively active form of the NFκB inhibitor, exhibited severely impaired establishment of latency in the spleen [72].

Since TLR9 in particular was reported to sense MHV-68 directly [153], we speculated that activation of TLR pathways by viral infection itself might contribute to the NFκB activation necessary for successful establishment and maintenance of latent infection. Indeed, downregulation of TLR9, but not TLR7, in S11 cells reduced NFκB activation in S11 cells and led to increased reactivation *in vitro*. Due to the relatively high rate of spontaneous reactivation in S11 cells and the resulting high levels of viral particles in the supernatant, we were not able to discern if viral particles in the supernatant or latent viral genomes are responsible for the intrinsic activation of the TLR9 pathway. Nevertheless, our observations indicate that MHV-68 has developed a strategy to usurp innate immune sensing to create a favorable environment for latent infection and use it to its own advantage. *In vivo*, suppression of lytic MHV-68 genes mediated by NFκB induction during *de novo* infection might serve as a negative feedback loop with the potential to limit productive replication and initiate latency. Additionally, such a mechanism could function as a homeostatic regulator to locally limit reactivation from latency. While the frequency of latently infected splenocytes in *tlr9*$^{-/-}$ or *tlr7*$^{-/-}$ mice did not differ significantly from the wild-type controls, spontaneous reactivation events upon explant were more frequent in *tlr9*$^{-/-}$ B-cells, confirming that intact TLR9 signaling contributes to limit lytic replication. Importantly, other gamma-herpesviruses use activation of NFκB in the host cell to help latency establishment as evidenced by EBV's binding to the cellular receptor CD21, which induces NFκB that in turn mediates activation of the viral latent gene promoter [154].

The finding that repeated or chronic stimulation of innate immune receptors can promote latent gamma-herpesvirus infection is particularly relevant when considering the role

of co-infections in the development of EBV-associated lymphoma. As shown before, repeated or continuous stimulation of TLRs during persistent infection *in vivo* increases the number of latently infected B-cells in mice. Notably, the numbers of EBV-infected B-cells in the blood of patients from regions holoendemic for malaria and with high incidence of BL are elevated in a similar fashion [155]. It is therefore conceivable that the repeated or constant immune activation caused by chronic malaria is an important factor in the etiology of eBL. In order to investigate this hypothesis, we started by testing if hemozoin, a putative TLR9 ligand released into the blood-stream of patients with *P. falciparum* infection has the potential to suppress lytic reactivation of EBV and MHV-68 *in vitro* (see manuscript 2). Our finding that natural hemozoin is able to suppress lytic viral gene expression in both viruses similar to TLR9 ligand CpG suggests that high concentrations of hemozoin in the blood-stream might indeed explain the increased numbers of infected B-cells observed in the blood of human patients. The existence of *Plasmodium* species pathogenic for laboratory mice makes it possible to test this hypothesis in an *in vivo* setting in the future.

Taken together, our results emphasize an important role of innate immune receptor signaling in promoting persistent infection of MHV-68 in B-cells and shows for the first time that TLR9 triggering by a gamma-herpesvirus itself might contribute to create a favorable host cellular environment for long-term latency. The impact of external TLR ligands on gamma-herpesvirus latency as observed in the MHV-68 model system are important not only as a possible link between EBV-associated lymphoma and co-infections such as *P. falciparum,* but also in the light of TLR ligands being increasingly used as adjuvants in the treatment of infectious diseases, cancer or autoimmunity, a practice which might detrimentally affect latent gamma-herpesvirus infections if administered chronically.

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Manuscripts

1. Activation of NFκB via Endosomal Toll-like Receptors 7 or 9 Contributes to Limiting Murine Herpes Virus 68 Reactivation

Florian Haas, Kazuma Yamauchi, Monika Murat, Michele Bernasconi, Noboru Yamanaka, Roberto F. Speck, and David Nadal

Submitted for publication to Journal of Virology

2. Hemozoin Extracted from *Plasmodium falciparum* Cultures Suppresses Gamma-Herpesvirus Reactivation *in vitro*

Florian Haas, Michele Bernasconi, Hans-Peter Beck, Cristina Nevado, and David Nadal

Manuscript in preparation

Activation of NFκB via Endosomal Toll-like Receptors 7 or 9 Contributes to Limiting Murine Herpes Virus 68 Reactivation

Florian Haas[a,b,c], Kazuma Yamauchi[a,b,c,d], Monika Murat[a,b,c], Michele Bernasconi[a,b,c], Noboru Yamanaka[d], Roberto F. Speck[e], and David Nadal[a,b,c] #

[a] Experimental Infectious Diseases and Cancer Research, [b] Division of Infectious Diseases and Hospital Epidemiology, [c] Children's Research Center, University Children's Hospital of Zurich, University of Zurich, Zurich, Switzerland,
[d] Department of Otolaryngology-Head and Neck Surgery, Wakayama Medical University, Wakayama, Japan,
[e] Division of Infectious Diseases and Hospital Epidemiology, University Hospital Zurich, University of Zurich, Zurich, Switzerland.

Running title: TLR7 and TLR9 signaling limits lytic MHV-68

Address correspondence to Dr. David Nadal, Division of Infectious Diseases and Hospital Epidemiology, University Children's Hospital of Zurich, Steinwiesstrasse 75, CH-8032 Zürich, Switzerland. Phone +41 44 266 7562; FAX +41 44 266 8072
e-mail: david.nadal@kispi.uzh.ch

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Author Contribution:
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Abstract

Latent gamma-herpesvirus infection is associated with various B-cell lymphoma. In order to understand and possibly treat herpesvirus-associated malignancies, it is vital to understand the factors that control the balance between the two transcriptional states of gamma-herpesviruses, lytic replication and latency. In this study, we used murine gamma-herpesvirus (MHV)-68 as a model system to investigate how engagement of endosomal Toll-like receptors (TLR) impacts on reactivation *in vitro* and establishment of latent infection *in vivo*. We found that treatment with TLR7 ligand R848 and TLR9 ligand CpG ODN suppresses reactivation of MHV-68 *in vitro* and that this effect correlates with the ability to activate the transcription factor NFκB. *In vivo*, continuous stimulation of TLR7 by R848 treatment led to an increased frequency of infected splenocytes compared to mock-treated control. Downregulation of TLR9 by RNA interference *in vitro* led to reduced nuclear levels of NFκB p65 and consequently to increased spontaneous reactivation in MHV-68 latently infected cells, indicating that the TLR9 pathway contributes to limiting spontaneous reactivation events. Frequencies of infected splenic B-cells in *tlr7*$^{-/-}$ or *tlr9*$^{-/-}$ mice did not differ from their wild-type counterpart. However, infected B-cells from *tlr9*$^{-/-}$ mice showed a higher frequency of reactivation compared to wild-type or *tlr7*$^{-/-}$ in an *ex vivo* reactivation assay. Our results show a suppressive effect of TLR7 and TLR9 signaling on MHV-68 reactivation and demonstrate that TLR9 signaling pathway is constantly active in infected cells, thereby contributing to NFκB activity and suppression of lytic replication.

INTRODUCTION

Gamma-herpesviruses are double-stranded DNA B-lymphotropic viruses capable of establishing life-long latent infections. Epstein-Barr virus (EBV) is the most prominent human gamma-herpesvirus with a seroprevalence in the adult population of over 90%. Latent EBV infection is associated with B-cell malignancies including endemic Burkitt's lymphoma and Hodgkin lymphoma in immunocompetent patients as well as lymphoma in immunocompromised patients (54). EBV's oncogenic potential is demonstrated by transformation and induction of unrestricted proliferation of infected B-cells *in vitro* (20).

One of the hallmarks of all gamma-herpesviruses is their sporadic reactivation from latent infection with subsequent production and release of new infectious particles (41). Sporadic reactivation is essential to ensure transmission to a new host but at the same time needs to be tightly controlled since the viral gene products represent a target for host immunity (37).

Intrinsic and extrinsic factors are thought to control the balance between latent and lytic infection of gamma-herpesviruses. The innate immune system, in particular signaling via Toll-like receptors (TLRs), is thought to be essential in the balance between latency and reactivation (14, 16, 29, 56). TLRs are a family of pattern-recognition receptors (PRRs) that play a central role in innate immune activation (44).

In mammals, four members of the TLR family are expressed almost exclusively in intracellular compartments (TLR3, TLR7, TLR8, and TLR9) (44), where they function as sensors for bacterial or viral nucleic acids (25). The natural ligand of TLR3 is double-stranded RNA while TLR7 senses single-stranded RNA, and TLR9 detects unmethylated DNA containing CpG motifs. TLR8 is structurally closely related to TLR7 and independently recognizes single-stranded RNA (23) but is thought to be biologically inactive in mice, instead having a regulatory function in modifying expression and signaling of TLR7 (8). Subsequent to ligation of the receptor, signaling is forwarded via the recruitment of specific Toll/IL-1 receptor (TIR)-domain containing adaptor proteins, myeloid differentiation primary response protein 88 (MyD88) in the case of TLR7 as well as TLR9 (32) and TIR-domain containing adaptor inducing IFN-β (TRIF) in the case of TLR3 (53). Eventually, signaling via TLRs 3, 7, and 9 leads to activation of the nuclear factor-κB (NFκB) axis which triggers pro- and anti-inflammatory cytokines (3). Importantly, activation of NFκB has been demonstrated to be crucial for the establishment and maintenance of latent gamma-herpesvirus infection in distinct ways. First, high levels of NFκB subunit p65 inhibit activation of lytic gene promoters of several gamma-herpesviruses (4, 22) and second, recombinant murine gamma-

herpesvirus 68 (MHV-68) expressing the constitutively active form of the NFκB-inhibitor IκBα is impaired in its ability to establish latent infection *in vivo* (28). Nevertheless and controversially, triggering of TLR3 and TLR9 were found to induce reactivation of MHV-68, while TLR7 did not (14), despite TLR7 and TLR9 sharing the same signaling pathway and even though TLR3, TLR7, and TLR9 are canonical activators of NFκB..

Many viruses including herpesviruses have evolved strategies to benefit from NFκB activation either directly by having NFκB binding sites in their promoters or indirectly by using the enhanced proliferation and survival of the host cell due to NFκB signaling to their advantage (39). TLR9 senses gamma-herpesviruses (12, 35, 51) and other herpesviruses (27, 55) directly, but the implication of a potential NFκB activation via TLR9 signaling caused by infection with a gamma-herpesvirus itself has not been examined in detail. TLR7 was shown to recognize ssRNA viruses (9, 30), but also seems to contribute to the sensing of gamma-herpesviruses in dendritic cells (35), likely mediated by viral RNA products.

In the present study, we used MHV-68 to study the effect of endosomal TLR triggering and activation of NFκB on the expression of lytic viral genes and shedding of viral particles *in vitro* and on the establishment of latent infection *in vivo*. MHV-68 is commonly accepted as a model system to investigate gamma-herpesvirus infection *in vivo*, since it is highly homologous to the human gamma-herpesviruses EBV and Kaposi sarcoma-associated Herpesvirus (KSHV) (48). We aimed at advancing the mechanistic understanding of the impact of TLR stimulation on spontaneous reactivation from latent infection using a cell line harboring latent MHV-68. Further, we tested whether TLR7 or TLR9 signaling contributes to NFκB activation in latently MHV-68-infected cells and impacts on viral behavior both *in vitro* and *in vivo*.

MATERIAL AND METHODS

Ethics Statement. All animal experiments were done according to the guidelines of the Animal Welfare Act provided by the Swiss Federal Veterinary Office (www.bvet.admin.ch), and by the veterinarian authorities of the Canton of Zurich, Switzerland approved all procedures (License Nr. 100/2012).

Cell culture and MHV-68 viral production. The MHV-68-infected murine B-cell line S11 (courtesy of Prof. Ren Sun, Los Angeles, CA) was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% penicillin-streptomycin and 2mM L-glutamine; baby hamster kidney cells (BHK-21) (6) (courtesy of Prof. A. Nash,

University of Edinburgh, UK) were cultured in Glasgow minimal essential medium (GMEM) supplemented with 10% Tryptose Phosphate Broth, 10% heat-inactivated FCS and 1% penicillin-streptomycin; murine fibroblast cells NIH3T12 (courtesy of Prof. S. Speck, Emory Vaccine Center, GA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FCS, 1% penicillin-streptomycin and 2mM L-glutamine (all reagents from Invitrogen, Basel, Switzerland).

Wild type MHV-68 clone g2.4 (11) (courtesy of Prof. Ren Sun, Los Angeles, CA) was grown in BHK-21 cells (43). Cells and supernatant were harvested at 7 dpi, centrifuged at 1,400 rpm for 5 min, and the supernatant was passed through a TPP PES 0.22μm filter (Omnilab, Mettmenstetten, Switzerland) and stored at -80°C. MHV-68 titers were determined by plaque assay on BHK-21 cells.

Mice. C57BL/6 mice were purchased from Harlan (The Netherlands). $Tlr7^{-/-}$ and $tlr9^{-/-}$ mice on a C57BL/6 background were obtained from the Swiss Immunological Mutant Mouse Repository (SwiMMR, Zurich, Switzerland). All animals were kept in a specific pathogen free environment.

Detection of *tlr* gene expression. Gene expression of *tlr*s was determined by reverse transcription PCR using gene specific primers as described in (10) and *gapdh* gene expression was used as a loading control.

TLR triggering. S11 cells were resuspended at 1×10^6 cells/ml of supplemented RPMI and stimulated with 25 μg/ml Poly(I:C), 3 μM of R848 or 0.5 μM of CpG ODN 1826 (Invivogen, San Diego, CA), 2 hours prior to stimulation with 10 ng/ml of 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich, Buchs, Switzerland). 24 hours after treatment, cell pellets were assayed for MHV-68 lytic gene expression by qPCR and supernatants were tested for MHV-68 production by plaque assay (see below).

Reverse transcription and quantitative PCR. Expression of MHV-68 lytic genes *ORF50*, *ORF21* and *M7* was determined by quantitative polymerase chain reaction (qPCR) using ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Rotkreuz, Switzerland). Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland) and contaminating DNA was removed by DNAse treatment (DNA-free Ambion; Applied Biosystems, Rotkreuz, Switzerland). cDNA was synthesized using High Capacity cDNA

Reverse Transcription Kit (Applied Biosystems). Amplification of synthesized cDNA was performed using TaqMan® Gene Expression Master Mix (Applied Biosystems). The primers and probe sequences used for MHV-68 genes are indicated in Table S1. Primers and probes for *tlr7* (Mm00446590_m1), *tlr9* (Mm00446193_m1), *ifnb1* (Mm00439552_s1), *il-6* (Mm99999064_m1), *il-10* (Mm00439614_m1) and *gapdh* (Mm99999915_g1) were all from Applied Biosystems.

Plaque assay. MHV-68 titers in supernatant of S11 cells were determined by plaque assay as described (43). BHK-21 cells were plated onto 6-well plates at 2×10^5 cells/well one day prior to infection. Serially diluted viral supernatants were placed onto monolayers and incubated for 1h at 37ºC in 5% CO_2. Supernatants were then removed and replaced with complete GMEM containing 1% methyl cellulose (Sigma-Aldrich). After 3-4 days, the monolayers were fixed with methanol, stained with neutral red solution (Sigma-Aldrich) and the numbers of plaques were counted.

Preparation of nuclear extracts. 2×10^6 cells were washed with Tris-buffered saline (TBS) and cell pellet was resuspended in 800 µl cold cell lysis buffer (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF), kept at 4ºC for 15 min, and vortexed for 10 seconds after 50 µl of a 10% solution of Nonidet P-40 (Roche, Mannheim, Germany) was added. The homogenate was centrifuged for 30 seconds, the nuclear pellet was re-suspended in 100 µl ice-cold nuclear lysis buffer (20 mM HEPES pH 7.9; 0.4 M NaCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF) and rocked at 4ºC for 15 min on a shaking platform. The nuclear extract was centrifuged for 5 min at 4ºC and the supernatant was stored at -80ºC.

Western blot analysis. Nuclear extracts were analyzed by SDS-PAGE on a NuPAGE 10% Bis-Tris gel (Invitrogen). The proteins were transferred onto PROTRAN Nitrocellulose Transfer Membrane (Whatman, Kent, UK) and probed with specific antibodies against NFκB p65 (sc-8008, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), PCNA (sc-25280; Santa Cruz) and beta-actin (Cat. No. 4967, Cell Signaling Technology, Beverly, MA). GeneTools software (SynGene, Cambridge, UK) was used for densitometric analysis.

Detection of spliced variant of *ORF73*. RNA isolation and cDNA synthesis were performed as described above. 2 µl of each cDNA reaction was used as template in a nested PCR

reaction specific for *ORF73* spliced transcripts as described (1), and products of the reaction were separated on a 1.5% agarose gel. cDNA from S11 cells was used as a positive control, and cDNA generated from uninfected splenocytes was used as a negative control.

Mice infection, R848 treatment, and limiting-dilution nested PCR. Mice were infected by injecting 10^5 pfu of MHV-68 i.p. in 100µl PBS. Daily treatment was done with 1mg/kg body weight R848 in 100µl PBS based on previous work (2), or PBS alone by i.p. injection for 19 days. At 20 dpi, mice were sacrificed by inhalation of CO_2, and splenocytes were isolated as described above. The frequency of MHV-68 genome-positive cells was determined by a previously published method (45). Nonlinear regression curve was fit using GraphPad Prism software (La Jolla, CA). Frequencies were calculated as the splenocyte number at which 63.2% of reactions were positive based on Poisson distribution.

TLR downregulation by RNA interference. S11 cells were transfected with psiRNA-h7SK plasmids expressing shRNA targeting *tlr7*, *tlr9,* or Luciferase gene, respectively, as well as green-fluorescent protein (GFP) and Zeocin resistance from a separate promoter (Invivogen, San Diego, CA). Transfection was performed using NEON® Transfection System (Invitrogen, Carlsbad, CA) according to the manufacturers instructions. Transfected cells were selected in complete RPMI containing 200 µg/ml Zeocin (Invivogen, San Diego, CA) and successful selection was confirmed by flow cytometry.

***Ex vivo* MHV-68 reactivation assay.** Mice were sacrificed by terminal anesthesia using a CO_2 chamber. Whole spleens were harvested, transferred to ice-cold PBS and passed through a 70 µm nylon mesh cell strainer (BD Falcon, BD Biosciences, Chicago, IL). Erythrocytes were removed by ammonium chloride lysis solution (Stemcell Technologies, Grenoble, France). B-cells were isolated by negative selection using a murine B-cell isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified B-cells were counted and resuspended in a two-fold dilution series starting at 10^6 cells/ml. Dilutions were plated on monolayers of NIH3T12 murine fibroblasts in a 96-well plate, 100 µl/well and 12 wells per dilution. As a control, uninfected B-cells were plated in 12 wells at 10^5 cells per well. 9 days later, wells were inspected for cytopathic effect by microscopy and the frequency of reactivation was calculated based on Poisson distribution.

Statistical analysis. Data were statistically analyzed with the unpaired *t*-test using GraphPad Prism software (La Jolla, CA). *P* values below 0.05 were considered statistically significant.

RESULTS

Stimulation of endosomal TLR7 and TLR9 but not TLR3 causes nuclear accumulation of NFκB p65 in S11 cells

To investigate the effects of TLR triggering on latent gamma-herpesvirus infection *in vitro*, we used the latently MHV-68-infected murine B-lymphocyte cell line S11. S11 was isolated from a lymphoma in a BALB/c mouse infected with MHV-68; each S11 cell harbors reactivation competent MHV-68 (47).

First, we verified that S11 cells express signaling-competent TLR3, TLR7, and TLR9 using reverse transcription PCR (Fig. 1A). To confirm activation of NFκB upon TLR triggering, we treated S11 cells with ligands to TLR3, TLR7, and TLR9 and NFκB inhibitor Bay 11-7082, respectively, and assessed subsequent nuclear accumulation of NFκB subunit p65 at different time points by Western blotting (Fig. 1B, PCNA loading controls see Fig. S1).

Poly(I:C) did not lead to any accumulation of NFκB p65 in the nucleus. Conversely, R848 that triggers TLR7 and CpG ODN 1826 which triggers TLR9 lead to activation of NFκB. S11 cells treated with R848 or CpG ODN 1826 showed stronger nuclear accumulation of NFκB p65 compared to untreated cells for at least 24 h after treatment (Fig. 1B). By contrast, NFκB inhibitor Bay 11-7082 reduced nuclear NFκB p65 to minimal levels at 1.5 h after treatment (Fig. 1B).

To verify signaling competence, we measured the induction of cytokine expression in response to TLR ligands by qPCR (Fig. 1C). TLR3 ligand Poly(I:C) robustly induced expression of IFN-β1 (Fig. 1C, left panel), most likely by activating Interferon regulatory factor 3 (IRF3) pathway (53) while R848 and CpG ODN 1826 did not. On the other hand, R848 and CpG ODN 1826 induced expression of the cytokines IL-6 (Fig. 1C, middle panel) and IL-10 (Fig. 1C, right panel) while Poly(I:C) did not.

These results demonstrate that endosomal TLRs activate distinct signaling pathways resulting in differential induction of transcription factors in the S11 model cell line. While the stimulation of TLR7 and TLR9 leads to activation of NFκB and subsequent induction of IL-6 and IL-10, stimulation of TLR3 does not activate NFκB but rather induces expression of IFN-β, probably via IRF3 transcription factor.

Stimulation of TLR7 and TLR9 but not TLR3 suppresses both spontaneous and induced MHV-68 reactivation in S11 cells

A fraction of S11 cells constantly undergoes spontaneous reactivation from latent MHV-68 infection as evidenced by expression of *ORF50*, the initiator of lytic replication (52) as well as shedding of infectious MHV-68 particles to the supernatant. The frequency of reactivation of MHV-68 can be increased by treatment with 12-O-tetradecanoyl-13-phorbolacetate (TPA) (47).

To monitor the impact of endosomal TLR triggering on MHV-68 reactivation, S11 cells were treated with Poly(I:C), R848 and CpG ODN 1826, respectively, for 24 h before analyzing the mRNA expression levels of immediate-early lytic gene *ORF50* (Fig. 2A, black bars), early lytic gene *ORF21* and late lytic gene *M7* (31) by qPCR as well as measuring infectious MHV-68 particles in the supernatant by plaque assay (Fig. 2A, white bars). Treatment with 25 μg/ml Poly(I:C) did not change the expression of *ORF50* or the release of infectious particles. Treatment with 3 μM R848 or 0.5 μM CpG ODN 1826, however, decreased the levels of all lytic genes compared to the mock-treated control (Fig. S2), which was compatible with suppression of the entire MHV-68 lytic transcription program. Consequently, the titer of infectious MHV-68 in the supernatant decreased by about 50% compared to the mock-treated control. Addition of 10 ng/μl TPA to the culture medium for 24 h increased the mRNA levels of *ORF50* about 4-fold and MHV-68 particle production about 2-fold compared to the control. When the cells were treated with R848 or CpG ODN 1826 2 h prior to adding TPA, the pro-lytic effect was abolished. Taken together, these results indicate that signaling by TLR7 and TLR9 suppresses both spontaneous as well as induced reactivation of the lytic cycle while TLR3 signaling has no effect.

Next, we wanted to see whether the suppressive effect of TLR7 and TLR9 stimulation is dependent on their ability to activate NFκB. To this end, we treated S11 cells with 1 μM NFκB inhibitor Bay 11-7082 2 h prior to the stimulation with TLR ligands and analyzed lytic reactivation 24 h later (Fig. 2B). We found that pre-treatment with Bay 11-7082 completely abolished the suppressive effect of CpG ODN 1826 and partially abolished the effect of R848, indicating that the impact of TLR triggering on lytic replication is at least partially dependent on NFκB activation. In the absence of TLR stimulation, the treatment with Bay 11-7082 led to a slight increase in lytic reactivation compared to mock-treated control, in good agreement with earlier reports in which Bay 11-7082 provoked reactivation of the human gamma-herpesviruses EBV and KSHV from latently infected B-cells *in vitro* (4, 17).

Stimulation of TLR7 in vivo leads to increased frequency of infected splenocytes after intraperitoneal injection of MHV-68

Based on our *in vitro* data, and since NFκB activation was shown to be important for the establishment of latent MHV-68 infection *in vivo* (28), we hypothesized that TLR7-mediated activation of NFκB would support the establishment of MHV-68 latency *in vivo* in a similar fashion as shown for TLR9 (14).

To test this hypothesis, we infected 10 C57BL/6 mice intraperitoneally (i.p.) with $1x10^5$ pfu MHV-68. We chose i.p. infection since this route seeds MHV-68 directly to the spleen without the need of amplification through lytic replication (49), therefore allowing the virus to reach the main site of latent infection directly. One day post inoculation (dpi) the mice were segregated into two groups and treated daily with either 1 mg/kg R848 in 100 µl of PBS or PBS alone (mock) i.p. for 19 days. Mice were sacrificed at 20 dpi, a time when lytic MHV-68 infection has been cleared from the spleen and predominantly latent infection can be expected (49).

To investigate whether treatment with R848 following inoculation with MHV-68 facilitates establishment of latent infection we isolated splenocytes from mice at 20 dpi and subjected them to analysis by limiting dilution nested PCR targeting MHV-68 gene v-cyclin (*ORF72*). We found that splenocytes from mice treated daily with R848 showed a 7-fold higher frequency of cells positive for MHV-68 genome than splenocytes from mice mock-treated with PBS (1 in 100 vs. 1 in 750; Fig 3A).

To confirm latent MHV-68 infection, we tested the splenocytes for expression of spliced variant of *ORF73* (mLANA), a transcript associated with MHV-68 latency (1), by reverse transcription PCR (Fig. 3B). At 20 dpi, *ORF73* spliced transcripts were detected in all samples from mice treated with R848 but inconsistently in samples from mock treated mice, reflecting the lower frequency of splenocytes harboring latent MHV-68.

Thus, TLR7 triggering clearly promotes establishment of infection and the size of latent MHV-68 reservoir.

Silencing of TLR9 but not TLR7 leads to decreased levels of nuclear NFκB and enhanced reactivation in S11 cells

Latently MHV-68-infected S11 cells show a basal level of NFκB activity (Fig. 1B). Since nucleic-acid binding endosomal TLRs have been implicated in the sensing of various viral infections and TLR7 and TLR9 both have the ability to activate NFκB, we aimed at

investigating whether the basal activity of NFκB is mediated by TLR sensing of MHV-68 infection.

To assess the role of TLR7 and TLR9 in this context, S11 cells were transfected with plasmids expressing shRNA against TLR7, TLR9, or luciferase as control. Antibiotic selection yielded stable cell lines with >95% of the cells expressing the GFP cassette included in the vector containing the shRNA (Fig. S3). Stably transfected cells showed a reduction in TLR7 mRNA of 70% and a reduction of TLR9 mRNA of 55%, respectively, as measured by qPCR (Fig. 4A). S11 cell lines genetically complemented with shRNA against luciferase (shLuc) did not have any effect on TLR expression and served as control.

Levels of NFκB p65 was similar in cells with reduced expression of TLR7 as in parental S11 cells as well as in the control cell line shLuc. In cells with reduced TLR9 expression however, lower levels of nuclear NFκB p65 were detected. We therefore hypothesize that latent MHV-68 infection causes a constant low-level activation of TLR9 but not TLR7 signaling pathway culminating in the nuclear translocation of NFκB p65, thereby contributing to a basal NFκB activation.

Since in previous experiments a reduction in nuclear NFκB p65 by the inhibitor Bay 11-7082 resulted in increased reactivation and viral production (Fig. 2B), we tested whether the stable cell lines silenced for TLR7 or TLR9 differed in their general propensity to spontaneous reactivation compared to the parental cell line. Quantification of lytic viral gene expression by real-time PCR and of infectious viral particle production by plaque assay revealed a 2-fold increase in expression of *ORF50* (Fig. 4C, black bars) as well as a 2-fold increase in the concentration of viral particles in the supernatant (Fig. 4C, white bars) of cell lines silenced for TLR9 but not of cell lines silenced for TLR7 or of the control cells expressing shRNA against luciferase.

These results suggest a contribution of continuous TLR9 signaling to basal NFκB activation levels and suppression of spontaneous reactivation.

Lack of TLR9 does not impact early establishment of infection *in vivo* but favors reactivation of primary B-cells *ex vivo*

Since activation of the TLR7 and TLR9 pathways support the establishment and maintenance of latent MHV-68 infection in splenocytes and since MHV-68 infection itself might promote latency via triggering TLR9, we hypothesized that lack of TLR9 would result in a reduced latent reservoir of MHV-68. Thus, we infected i.p. wild-type (wt), *tlr7*$^{-/-}$ and *tlr9*$^{-/-}$ mice (each of the genotypes n=15) with MHV-68; as performed, focusing on the *de novo* establishment

of infection in splenic B-cells, the consensus primary site of MHV-68 latency. None of the mice showed signs of distress or illness throughout the course of the experiment and all animals were sacrificed at 7 dpi.

We did not find significant differences in the frequency of infected B-cells between any of the three groups (wt: 1 in 2,700; *tlr7*$^{-/-}$: 1 in 3,300; *tlr9*$^{-/-}$: 1 in 4,200) performing limiting-dilution nested PCR on purified B-cells (Fig 5A). We then assessed *ex vivo* reactivation of B-cells by limiting-dilution reactivation assay and found a higher frequency of reactivating *tlr9*$^{-/-}$ B-cells compared to wt or *tlr7*$^{-/-}$ (wt: 1 in 950,000; *tlr7*$^{-/-}$: 1 in 1,000,000; *tlr9*$^{-/-}$: 1 in 370,000). These findings are in line with both our *in vitro* results from silencing TLR7 or TLR9 in S11 cells as well as with the findings reported by Guggemoos et al. (18) who found higher titers of lytic MHV-68 in *tlr9*$^{-/-}$ mice than in wt mice after i.p. infection. Thus, it seems that impaired TLR9 signaling renders MHV-68-infected B-cells more likely to reactivate whereas impaired TLR7 signaling does not.

DISCUSSION

Activation of the innate immune system via TLRs largely impacts on gamma-herpesvirus latency and reactivation. We here studied the effect of endosomal TLR stimulation on latently infected cells *in vitro* and on the establishment of gamma-herpesvirus infection *in vivo* using the MHV-68 mouse model. We found that (i) signaling of TLR7 or TLR9 but not of TLR3 inhibit both spontaneous and induced reactivation in the latently MHV-68-infected B-cell line S11 and that this effect is, at least partially, dependent on the induction of NFκB; (ii) stimulation of TLR7 during infection increases the number of latently infected splenocytes; (iii) downregulation of TLR9 but not of TLR7 reduces basal NFκB activity and increases spontaneous MHV-68 reactivation in latently infected cells; and (iv) while the lack of TLR7 or TLR9 does not change the frequency of infected B-cells *in vivo*, MHV-68-infected B-cells from *tlr9*$^{-/-}$ mice show increased propensity to reactivate *ex vivo*. Our results unprecedentedly show that activation of NFκB via TLR7 signaling profoundly impacts the establishment and maintenance of latent MHV-68 infection, as is the case for TLR9 signaling. In absence of external ligands, constant activation of TLR9 caused by persistent MHV-68 infection might contribute to robust activation of NFκB, thus creating a favorable environment for latency.

Our *in vitro* experiments using S11 cells as a model for established MHV-68 latency invariably showed that activation of the TLR9 pathway suppresses spontaneous as well as induced MHV-68 reactivation (Fig. 2A). It seems likely that this feature is shared by other gamma-herpesviruses, as previous studies in our lab demonstrated that stimulation by CpG

ODN similarly leads to a suppression of *BZLF1* expression (29, 56), EBV's master regulator lytic gene (46), in Burkitt's lymphoma cells. Our finding that signaling via TLR7 suppresses MHV-68 reactivation is unprecedented. It seems plausible that TLR7 and TLR9 would have a comparable effect when stimulated, since they share a signaling pathway via the common adaptor molecule MyD88. Upon stimulation of either receptor we observed comparable activation of NFκB, albeit with slightly distinct kinetics (Fig. 1B). The facts that NFκB inhibitor counteracts the suppression by TLR ligands and in former studies NFκB was shown to suppress the lytic gene promoters of MHV-68 as well as the human gamma-herpesviruses EBV or KSHV (4, 22) support the hypothesis that downregulation of lytic MHV-68 upon TLR7 or TLR9 stimulation in S11 cells is due to activation of NFκB. Nevertheless, we cannot exclude that triggering of endosomal TLRs might activate cellular signaling pathways apart from NFκB that might impact on MHV-68 gene expression. *In vitro* data of Gargano et al. (14) showed that TLR3 or TLR9 triggering provoked lytic MHV-68 reactivation in the cell lines A20HE1 and A20HE2 while TLR7 triggering had no effect. The activation of NFκB was not tested. Thus, differences in endogenous levels of NFκB activation or TLR signaling competence between these cell lines might account for the divergent observations. Alternatively, the reason for the apparently contradictory observations may lie in the distinct origin of MHV-68 persistent infection. While S11 cells derive from a naturally MHV-68-associated lymphoproliferation, A20HE1 and A20HE2 cell lines derive from murine lymphoma cells secondarily infected *in vitro* with a recombinant strain of MHV-68 (13). In a different study, increased reactivation of KSHV from primary effusion lymphoma cells was shown upon treatment with ligands to TLR7/8 (16). The critical signaling pathway in these cells was shown to be dependent on IRF7, however, further demonstrating that the impact of TLR signaling on gamma-herpesvirus reactivation may depend on cellular context.

In vivo, recurrent activation of TLR7 signaling by treatment with R848 following *de novo* infection of wt mice led to a higher frequency of MHV-68 infected splenocytes compared to mock treatment (Fig. 3A). This observation is comparable with the findings of both Gargano et al. (14) as well as Ptaschinski et al. (34) who reported increased frequency of infected splenocytes *in vivo* upon stimulation of TLR9 by administration of CpG ODN. A higher frequency of cells positive for MHV-68 genome indicates an increase in the pool of latently MHV-68-infected cells and this is supported by the detection of MHV-68 latency-associated mLANA transcripts in our experiments (Fig. 3B). Failure to detect mLANA transcripts in two samples from untreated controls reflects the limit of detection of the nested PCR rather than absent infection, since splenocytes from all mice were positive for MHV-68

genome (Fig. 3A) but only 5-10% of latently infected cells are expected to express mLANA (1). Based on our observations *in vitro*, we hypothesize that both TLR7 and TLR9-induced activation of NFκB promotes maintenance and expansion of the latently infected B-cell pool. Notably, stimulation with TLR ligands has been shown to induce proliferation of murine B-cells (19) and is known to synergize with EBV in driving proliferation of EBV-infected human B-cells (21), which could explain elevated frequencies of infected cells without the need for lytic replication. Our hypothesis is supported by the absence of late lytic MHV-68 gene expression reported by Ptaschinski et al. (34). The importance of functional NFκB for the establishment of MHV-68 latency *in vivo* has been elegantly demonstrated by Krug et al. (28) who reported severe impairment in establishment of splenic latency by a recombinant strain of MHV-68 that expresses a constitutively active form of the NFκB inhibitor IκBαM. Furthermore, NFκB signaling has been shown to be an important factor for the survival of lymphoma cells latently infected with KSHV (26) and EBV-transformed B-cells *in vitro* (5).

We observed a constant activation of the NFκB pathway in the model cell line S11 as evidenced by high basal levels of NFκB subunit p65 in the nucleus (Fig. 4B), which drives survival and proliferation (24). NFκB levels in the nucleus were reduced by stable downregulation of TLR9 but not of TLR7 expression by RNA interference which consequently led to an increased rate of spontaneous reactivation (Fig. 4C), implying a constant stimulation of TLR9 in infected S11 cells. Spontaneous reactivation of MHV-68 is more frequent in S11 cells than in MHV-68-infected B cells *in vivo* and therefore the effect of TLR9-mediated NFκB activation might be rather high in comparison to latent infection *in vivo*. Nevertheless, it has been shown by various groups that TLR9 is triggered upon infection by herpesviruses (36, 40), including members of the gamma-herpesvirus family EBV (12) and KSHV (50). Suppression of lytic viral genes mediated by NFκB induction upon infection might serve as a negative feed-back loop with the potential to limit productive replication and initiate the latent state during acute infection *in vivo*. Additionally, such a mechanism could function as a homeostatic sensor to locally limit reactivation from latency.

Unlike TLR9, TLR7 does not contribute to NFκB activation in S11 cells in the absence of external stimulation. The main function attributed to TLR7 is the sensing of RNA viruses such as vesicular stomatitis virus and influenza (30). Only in one case is TLR7 reported to recognize the herpesvirus murine cytomegalovirus (57), a study, however, done in the context of plasmacytoid dendritic cells rather than B-cells.

In the context of *de novo* infection *in vivo*, our experiments with mice lacking either TLR7 or TLR9 did not show a significant difference in the frequency of infected splenic B-

cells at an early time following i.p. infection. It is possible that during prolonged infection, deficiencies in TLR signaling impact on the number of latently infected cells since a ten-fold decrease in infected splenocytes was reported in *myd88*$^{-/-}$ mice at 42dpi (15). While the numbers of infected B-cells in our experiments were comparable, B-cells from *tlr9*$^{-/-}$ mice showed a higher propensity to reactivate *ex vivo* while the *tlr7*$^{-/-}$ B-cells did not, in agreement with the results obtained with S11 cells.

Taken together, our results point towards an important role of TLR7- and TLR9-mediated activation of NFκB in promoting persistent infection of MHV-68 in B-cells and show that TLR9 triggering by persistent MHV-68 infection might contribute to maintaining NFκB activation during latency. Considering that we did not see a significant difference in the frequency of infected B-cells *in vivo*, it is conceivable that there are other systems in place contributing to NFκB activation, for example the retinoic-acid-inducible-gene (RIG)-I system that synergizes with TLR9 to induce the immune response against herpes simplex virus (36). Other gamma-herpesviruses use activation of NFκB in the host cell for benefit as evidenced by EBV's binding to the cellular receptor CD21, which induces NFκB that in turn mediates activation of the viral latent gene promoter (42) or the sustained moderate activation of NFκB reported upon infection of endothelial cells by KSHV that regulates viral gene expression(38).

Taken together, the latency-promoting effect of endosomal TLR-mediated NFκB activation reported here suggests a possible mechanism for the role of co-infections that are implicated in the pathogenesis of certain gamma-herpesvirus-associated malignancies. Indeed, with respect to EBV-associated malignancies, the parasite *Plasmodium falciparum* that causes malaria and is epidemiologically associated with endemic Burkitt's lymphoma was reported to directly stimulate TLR9 (7, 33). We showed that *P. falciparum* hemozoin suppresses lytic reactivation of EBV in Burkitt's lymphoma cells Akata (56), further underscoring a possible mechanistic link between malaria infection and the incidence of EBV-positive Burkitt's lymphoma. The situation in KSHV-associated primary effusion lymphoma, however, may be distinct as suggested by the lytic reactivation of KSHV following triggering of TLR7/8 (16). Nevertheless, our results are important in the light of TLR ligands being increasingly used as adjuvants in the treatment of infectious diseases, cancer or autoimmunity because of their potential to promote gamma-herpesvirus latency that may contribute to tumorigenesis.

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Figure 1

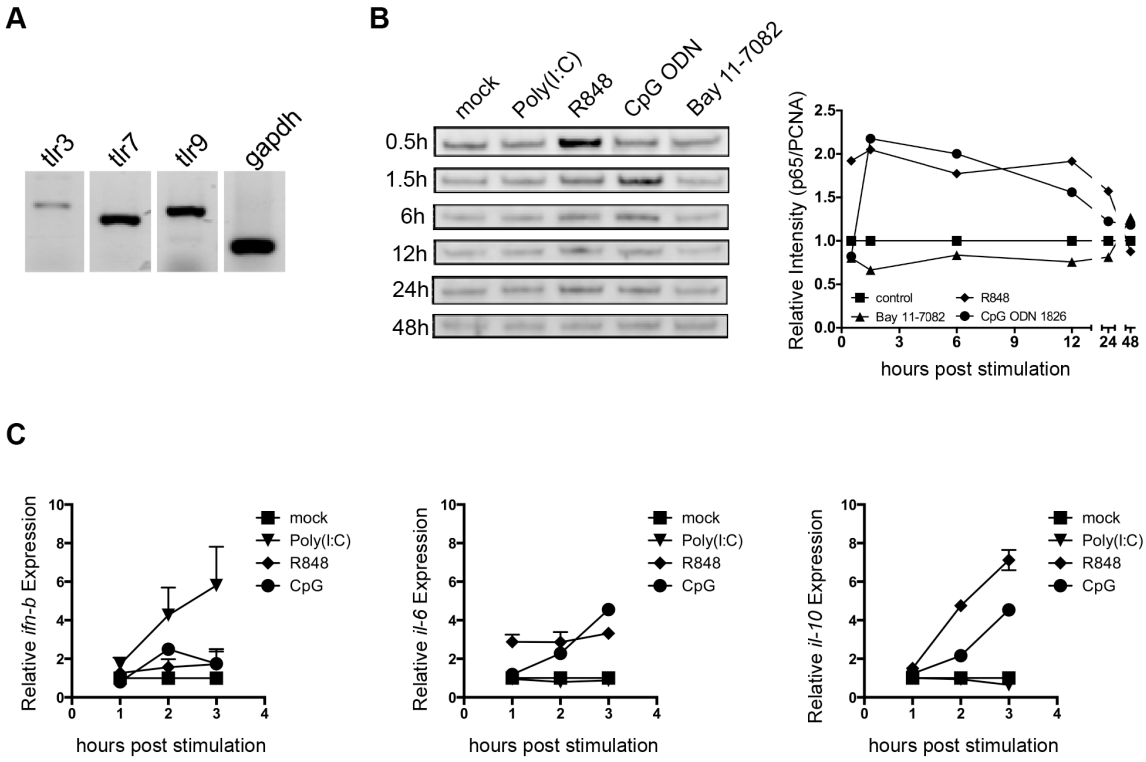

FIG 1 Triggering of endosomal TLR7 and TLR9 but not TLR3 activates NFκB in S11 cells.
(A) Expression of TLR3, TLR7 and TLR9 mRNA is detected in S11 cells by reverse transcription PCR.
(B) Triggering of TLR7 and TLR9 but not TLR3 activates NFκB in S11 cells. NFκB subunit p65 was detected in nuclear extracts of S11 cells at different timepoints after the treatment of mock (control), 25 µg/ml Poly(I:C), 3 µM R848, 0.5 µM CpG ODN 1826 or 1 µM NFκB inhibitor Bay 11-7082 by Western Blot. PCNA was used as a loading control and is shown in Fig. S1. The intensity of each band was analyzed densitometrically and normalized to respective PCNA levels (right side).
(C) Triggering of TLR3 induces a different pattern of cytokine expression than TLR7 or TLR9. S11 were stimulated with TLR ligands as done in (B) and harvested at different timepoints. Expression of IFN-beta, IL-6 and IL-10 was analyzed by qPCR, normalized to gapdh, and plotted relative to mock treated controls. Data shown in (C) are mean ± SD of three independent experiments. Some of the error bars are covered by the symbols.

Figure 2

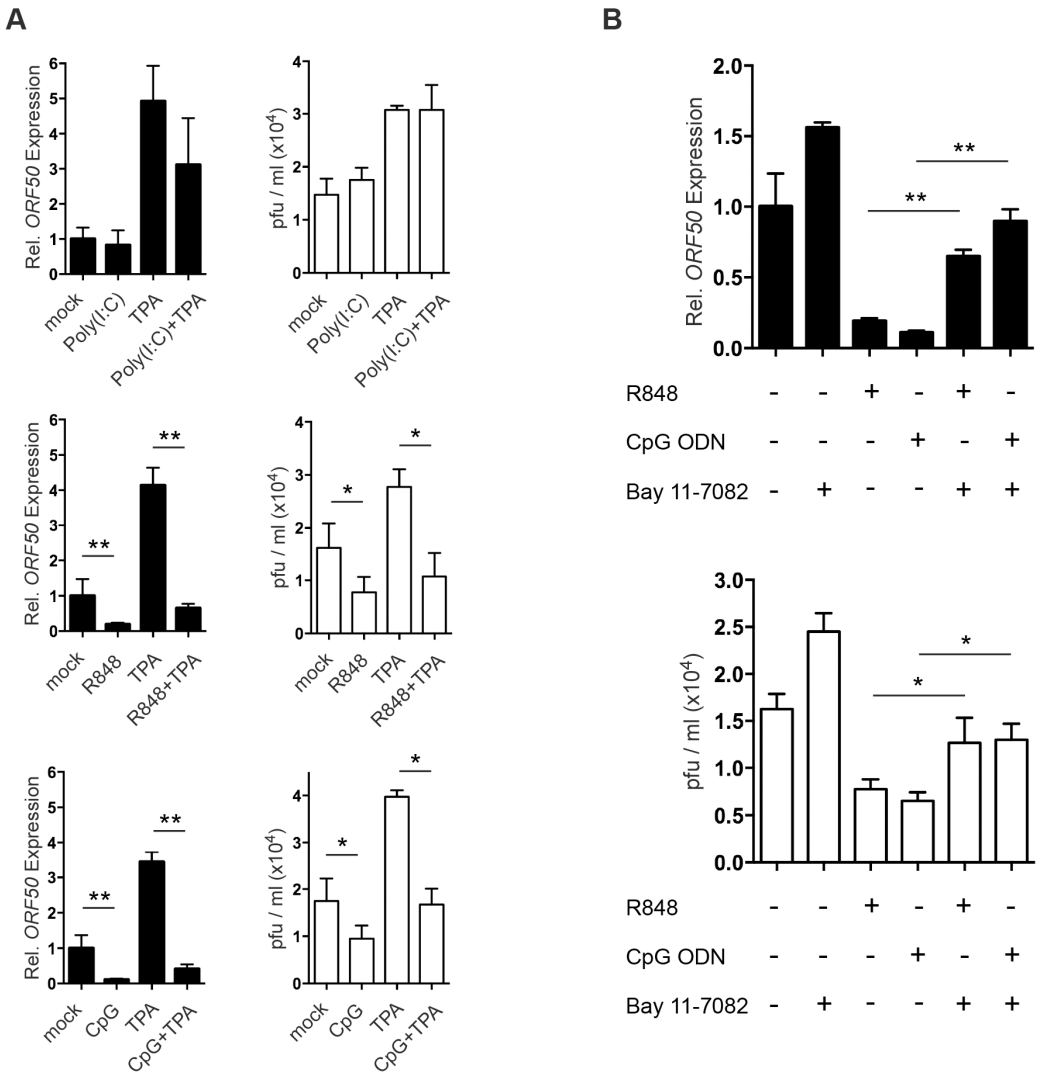

FIG 2 Triggering of endosomal TLR7 and TLR9 but not TLR3 inhibits expression of lytic MHV-68 genes and reduces viral particle shedding while TPA or NFκB inhibitor Bay 11-7082 increase spontaneous reactivation.
(A) S11 cells that are persistently infected with MHV-68 were mock treated or treated with TLR3 ligand Poly(I:C), TLR7 ligand R848, TLR9 ligand CpG ODN 1826, TPA or a combination of TLR ligand followed by TPA 2h later. 24h after the begin of treatment, expression levels of MHV-68 lytic gene ORF50 were analyzed by qPCR, normalized to gapdh, and plotted relative to untreated controls (black bars). Viral titers in the supernatants of treated cells were measured by Plaque Assay on BHK21 cells (white bars).
(B) S11 cells were mock treated or treated with R848, CpG ODN 1826,
NFκB inhibitor Bay 11-7082 or a combination thereof. 24h treatment, expression levels of MHV-68 lytic gene ORF50 was analyzed by qPCR (black bars). Viral titers in the supernatants of the treated cells were measured by plaque assay on BHK21 cells (white bars). Data shown in (A) and (B) are mean ±SD of three independent experiments. Statistics were calculated using unpaired t test (*, $p<0.05$; **, $p<0.01$).

Figure 3

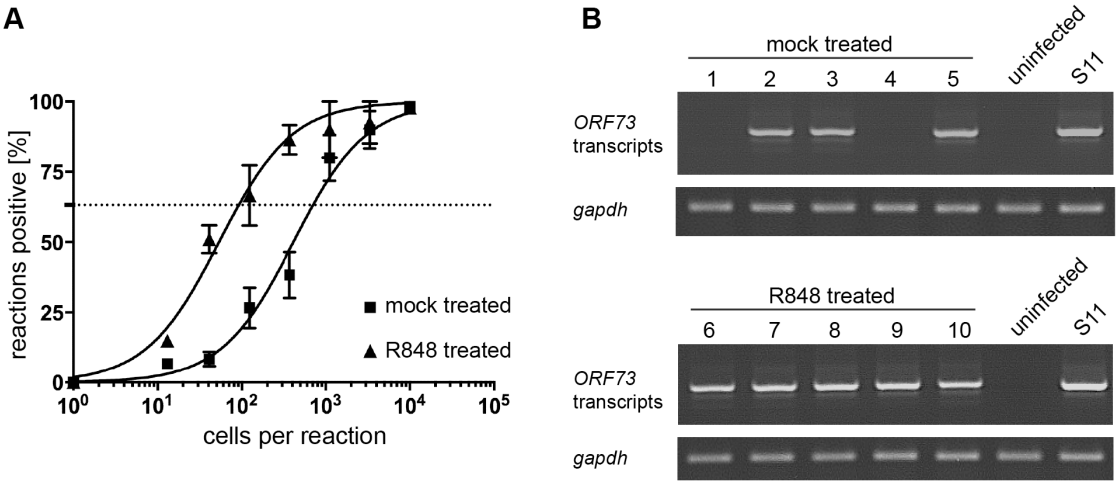

FIG 3 Treatment with TLR7 ligand R848 promotes establishment of latent MHV-68 infection in vivo. C57BL/6 mice were infected with MHV-68 by i.p. injection and treated daily i.p. with either 1mg/kg R848 in 100µl of PBS or PBS alone (mock) for 19 days. Treated and mock-treated groups contained 5 mice each.
(A) Splenocytes isolated from infected mice at 20dpi were analyzed by limiting-dilution nested PCR targeting ORF72. Data are expressed as mean percentages of positive reactions ±SD. Sigmoidal dose-response curve was fit by nonlinear regression analysis using GraphPad software. From the equation of the curve, the intersection with the dotted line at 63.2% was calculated and the frequency of MHV-68 positive splenocytes was assessed based on Poisson distribution. The resulting curves were compared by Mann-Whitney test (mock-treated vs. R848-treated p=0.016).
(B) Primary splenocytes from in vivo MHV-68 infected mice express virus latency-associated gene ORF73 (mLANA). 1 µg of total RNA isolated from 1x10(5) bulk splenocytes from each infected animal was reverse transcribed and analyzed for spliced ORF73 transcripts by nested PCR. RNA from persistently MHV-68 infected S11 cells was used as positive control. Amplification of the housekeeping gene gapdh served as control for successful reverse transcription.

Figure 4

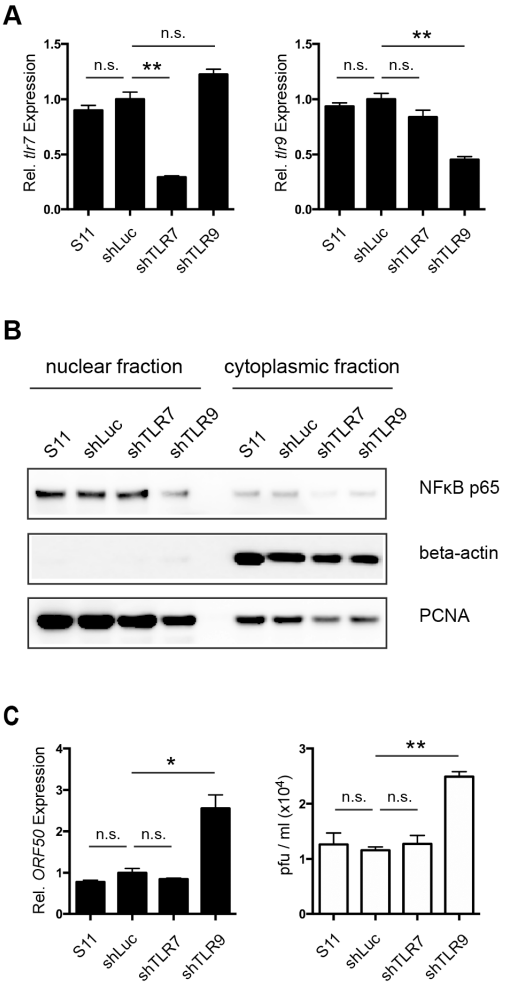

FIG 4 Downregulation of TLR9 but not of TLR7 leads to a decrease in nuclear NFκB p65 and increased spontaneous reactivation in S11 cells.
(A) Expression of TLR7 (left side) and TLR9 (right side) was analyzed by qPCR in parental S11 cells and stable cell lines containing plasmids expressing shRNA against Luciferase (shLuc), TLR7 (shTLR7) or TLR9 (shTLR9). Expression levels were normalized to gapdh and plotted relative to control plasmid shLuc.
(B) NFκB subunit p65, PCNA and beta-actin was detected in nuclear and cytoplasmic protein fractions from parental S11 and stably transfected cell lines by Western Blot.
(C) Expression of MHV-68 lytic gene ORF50 was analyzed by qPCR in parental S11 cells and stable cell lines shLuc, shTLR7 and shTLR9 (black bars). Expression levels were normalized to gapdh and plotted relative to control plasmid shLuc. Titers of infectious viral particles in the supernatant after 24h were quantified by plaque assay (white bars).
Data shown in (A) and (C) are mean ± SD of three independent experiments. Statistics were calculated using unpaired t test (*, $p<0.05$; **, $p<0.01$; n.s., $p>0.05$).

Figure 5

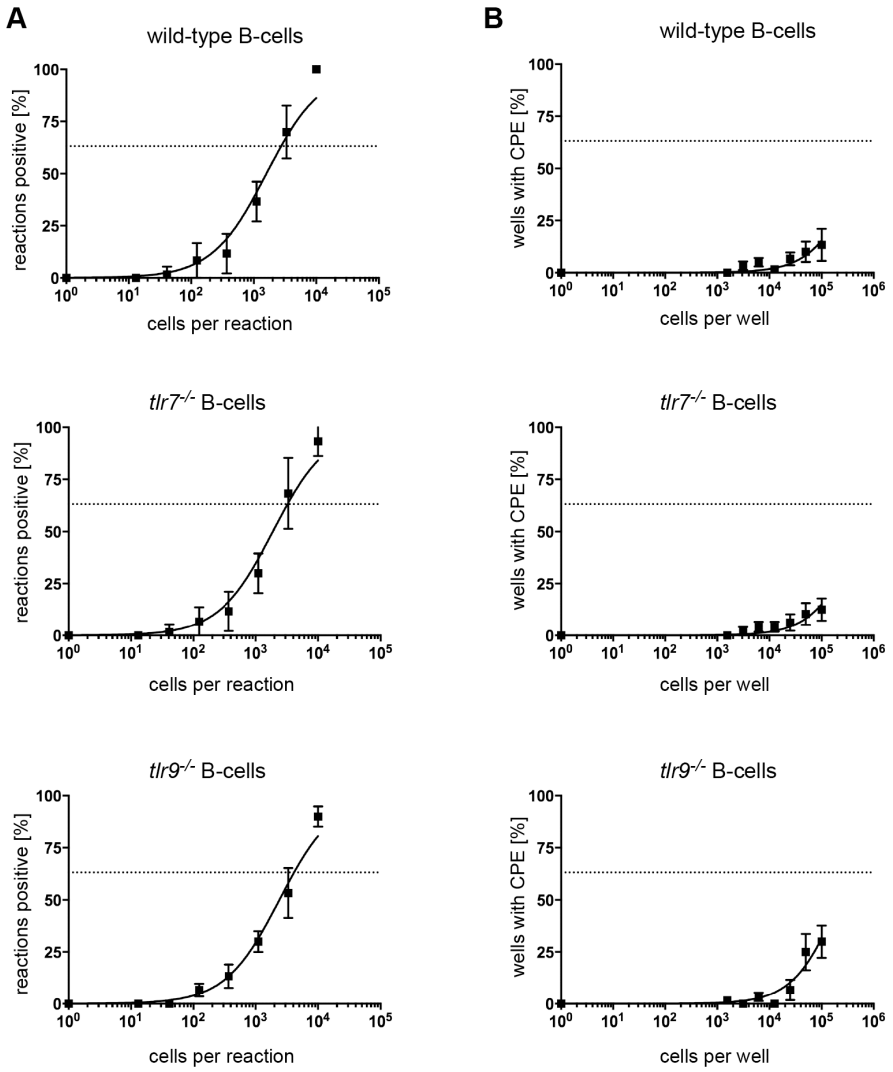

FIG 5 Lack of TLR7 or TLR9 does not impact early establishment of infection in B-cells upon i.p. injection in vivo but tlr9-/- B-cells show higher reactivation ex vivo. 15 mice each of the genotypes wt, tlr7-/- and tlr9-/- were infected with MHV-68 and sacrificed on day 7 post infection. Splenic B-cells were isolated by negative selection.
(A) Splenic B-cells from three mice of the same genotype were pooled and analyzed by limiting-dilution nPCR as in Fig.3. The resulting curves (5 per genotype) were compared by Mann-Whitney test and showed no significant difference between the genotypes (wt vs. tlr7-/- p=0.41; wt vs. tlr9-/- p=0.53; tlr7-/- vs. tlr9-/- p=0.67).
(B) Splenic B-cells were plated in two-fold dilution series on monolayers of susceptible fibroblasts. 9 days after seeding, the percentage of wells showing cytopathic effect was determined microscopically and plotted against the number of B-cells seeded. Extrapolation of the non-linear regression curve fit using GraphPad software was used to calculate the frequency of B-cells reactivating.
The resulting curves were compared by Mann-Whitney test (wt vs. tlr7-/- p>0.9; wt vs. tlr9-/- p=0.016; tlr7-/- vs. tlr9-/- p=0.016).

Supplementary Figure 1

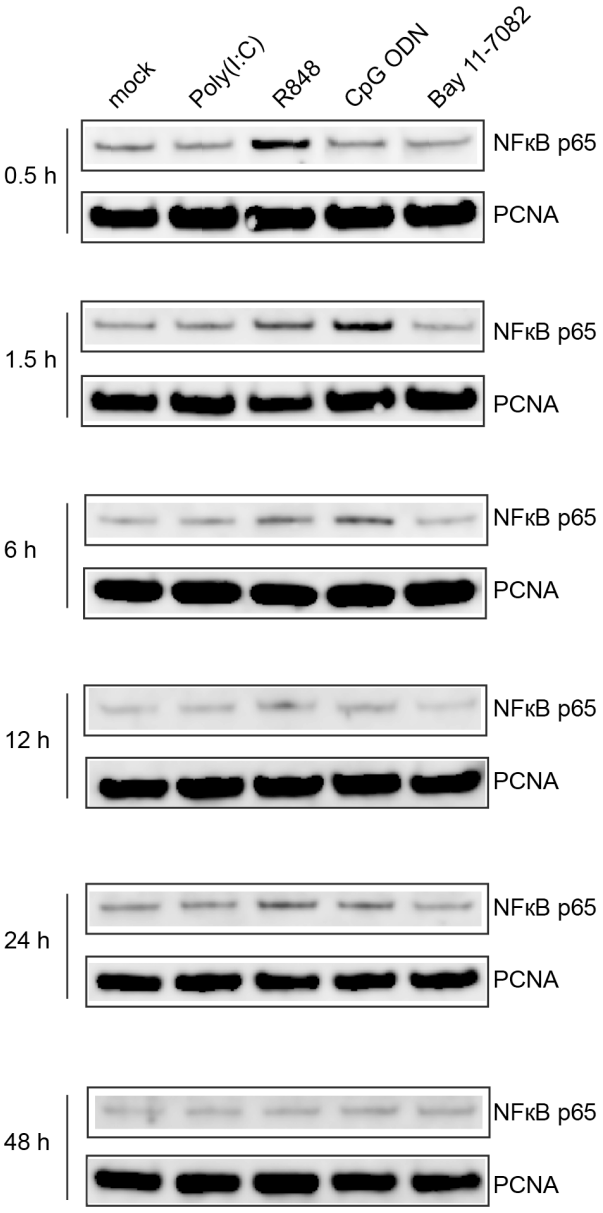

FIG S1. Triggering of endosomal TLR7 and TLR9 but not TLR3 activates NFκB in S11 cells. NFκB subunit p65 and nuclear protein PCNA were detected in nuclear extracts of S11 cells at different timepoints after the treatment of mock (control), 25 µg/ml Poly(I:C), 3 µM R848, 0.5 µM CpG ODN 1826 or 1 µM NFκB inhibitor Bay 11-7082 by Western Blot. PCNA was used as a loading control to normalize for protein input.

Supplementary Figure 2

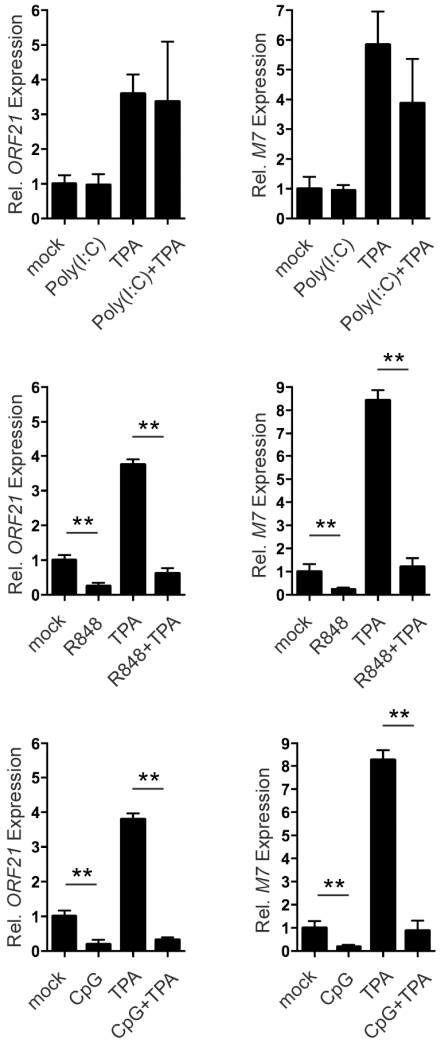

FIG S2 Triggering of endosomal TLR7 and TLR9 but not TLR3 inhibits expression of early and late lytic MHV-68 genes. S11 cells that are persistently infected with MHV-68 were mock treated or treated with TLR3 ligand Poly:IC, TLR7 ligand R848, TLR9 ligand CpG ODN 1826, TPA or a combination of TLR ligand followed by TPA 2h later. 24h after the beginning of treatment, expression levels of MHV-68 early lytic gene ORF21 and late lytic gene M7 were analyzed by qPCR, normalized to gapdh and plotted relative to mock-treated controls.

Hemozoin Extracted from *Plasmodium falciparum* Cultures Suppresses Gamma-Herpesvirus Reactivation *in vitro*

Florian Haas[a,b,c], Michele Bernasconi[a,b,c], Hans-Peter Beck[d], Cristina Nevado[e], and David Nadal[a,b,c]

[a] Experimental Infectious Diseases and Cancer Research, [b] Division of Infectious Diseases and Hospital Epidemiology, [c] Children's Research Center, University Children's Hospital of Zurich, University of Zurich, Zurich, Switzerland

[d] Swiss Tropical and Public Health Institute, Basel, Switzerland

[e] Organic Chemistry Institute, University of Zurich, Zurich, Switzerland

Author Contribution:

FH performed experiments shown in Fig1; Fig2; Fig3; Fig4 and wrote the manuscript

Abstract

Endemic Burkitt's Lymphoma is the most common pediatric cancer in equatorial africa. It is associated with gamma-herpesvirus Epstein-Barr virus and only occurs in regions where transmission of the malaria parasite *Plasmodium falciparum* is holoendemic, thus it is suspected to be a polymicrobial disease. Eventhough association with both EBV and malaria are established, the exact mechanism how chronic *P.falciparum* infection impacts on normally asymptomatic EBV and promotes incidence of EBV-positive cancer is unknown. Here we show that hemozoin, a waste product of parasite metabolism extracted from *P.falciparum* cultures *in vitro,* suppresses lytic reactivation from latency in both EBV-infected Burkitt's Lymphoma cells as well as mouse lymphoma cells infected with murine gamma-herpesvirus 68. Suppression of viral reactivation was only seen when cells were stimulated with natural hemozoin extract but not with synthetic analogue β-hematin. Suppression of lytic reactivation promotes latency and has been shown to increase the frequency of infected cells *in vivo*. We hypothesize that chronic malaria contributes to the incidence of eBL by suppressing EBV reactivation via constant shedding of hemozoin to the bloodstream and therefore increasing the pool of latently infected B-cells.

Introduction

Endemic Burkitt's Lymphoma (eBL) is the most common cancer in infants of equatorial Africa. The Irish surgeon Denis Burkitt first described this particularly fast growing B-cell malignancy in children in Uganda [1]. He established that eBL was restricted to climatic zones where the malaria parasite *Plasmodium falciparum* is holoendemic and concluded that eBL is associated with malaria [2,3]. Meanwhile, British Pathologist Anthony Epstein and his co-workers Bert Achong and Yvonne Barr identified virus particles resembling herpesviruses in cultured cells of a specimen of eBL from Uganda by electron microscopy [4]. The newly discovered virus was subsequently named Epstein-Barr virus (EBV) and turned out to be present in almost 100% of eBLs as well as in other cancers such as nasal carcinoma [5]. Further epidemiological evidence strongly suggested a causal relationship between EBV and eBL [6], even before the oncogenic potential of EBV, i.e., its ability to transform B-cells *in vitro* was demonstrated [7].

Both continuous exposure to the malaria agent *Plasmodium falciparum* and persistent infection with EBV have been identified as necessary co-factors for the development of eBL. Therefore, it seems likely that eBL should be considered a polymicrobial disease where chronic infection with *Plasmodium falciparum* is a prerequisite to create an environment in which an usually asymptomatic EBV persistence may turn into eBL. Yet, the tumorigenic pathogenetic mechanism of interaction between the virus and the parasite is unclear.

Several clinical studies have elaborated evidence for the impact of malaria on persistent EBV infection. Lam and colleagues [8] reported a 5-fold increase in the frequency of EBV-positive B-cells in the peripheral blood of children during acute malaria. These findings were confirmed by reports of increased EBV DNA levels in whole blood samples of pediatric patients from a region where malaria transmission is high compared to patients from a region where transmission is low [9], as well as 10-fold increase of EBV DNA load in peripheral blood mononuclear cells in children with malaria [10]. Thus, infection with *Plasmodium falciparum* seems to increase the number of latently EBV-infected B-cells and might therefore promote the risk for eBL.

Studies in our lab have shown that constant stimulation of the innate immune system via systemic administration of a synthetic ligand to Toll-like receptor (TLR)7 leads to a similar infection pattern with an increased frequency of latently infected B-cells in an *in vivo* infection model using murine gamma-herpesvirus 68 (MHV-68) (Haas, manuscript submitted). These results are in line with earlier studies reporting increased frequencies of infected splenocytes in MHV-68-infected mice following stimulation of TLR9 [11], which

shares a signaling pathway with TLR7. We further determined that stimulation of TLR7 and TLR9 suppresses MHV-68 reactivation *in vitro*. Therefore, we hypothesized that constant stimulation of TLR7 or TLR9 leads to increased frequencies of latently MHV-68-infected B-cells *in vivo* due to the reactivation-suppressing effect, in turn promoting latency over lytic replication and thus the survival of the infected host cell. Additionally, it is known that stimulation of TLR signalling pathways induces proliferation in B-cells [12], an effect likely contributing to the expansion of the reservoir of latently infected cells.

We found a similar reactivation-suppressing effect of TLR9 signalling in EBV-infected Burkitt's Lymphoma cells *in vitro* [13]. It is conceivable that continuous stimulation of TLR9 would expand the pool of infected B-cells *in vivo* in a similar fashion as seen in the mouse model. This raises the question whether the observed expansion of latently EBV-infected B-cells in the peripheral blood of patients chronically exposed to *Plasmodium falciparum* infection might be due to continuous stimulation of endosomal TLRs.

There is solid evidence that *Plasmodium falciparum* interacts with members of the TLR family. *Plasmodium* schizont extracts were demonstrated to stimulate dendritic cells via a TLR9-dependent pathway [14] and the *Plasmodium* waste product hemozoin was identified as a natural ligand for TLR9 [15]. Hemozoin is a crystalline compound formed in parasitized red blood cells in order to detoxify free heme that accumulates as the parasite digests hemoglobin [16]. Upon rupture of the red blood cell, hemozoin is released into the blood stream and potentially phagocytosed by immune cells. This makes hemozoin an interesting candidate for TLR9 stimulation in B-cells. Nevertheless, it is not undisputed whether hemozoin itself or associated parasite products are responsible for TLR stimulation. While several studies [15,17,18] claim that synthetic pure hemozoin crystals bind to and stimulate TLR9, findings by another group argue that it is not the hemozoin itself but rather hemozoin-associated *Plasmodium* DNA that elicits TLR9 responses [19].

Considering above commented literature, we hypothesize that recurring infection with *Plasmodium falciparum* contributes to eBL by promoting both proliferation and survival of latently EBV-infected B-cells via constant activation of TLR signalling, thus suppressing viral reactivation.

To test this hypothesis, we investigated whether *Plasmodium*-derived hemozoin or chemically synthesized hemozoin analog (β-hematin) acts as natural TLR9 ligand and reproduces the suppressive effect on gamma-herpesvirus reactivation that we found for canonical ligands to TLR9 such as CpG oligodenucleotides.

Results

Hemozoin isolated from P. falciparum cultures suppresses induced and spontaneous reactivation of gamma-herpesviruses

As we have shown previously, stimulation of gamma-herpesvirus infected B-cells with ligands to TLR9 can suppress viral reactivation by downregulating lytic viral gene expression. Since the malaria pigment hemozoin was reported to stimulate TLR9, we asked whether natural hemozoin from *P. falciparum* cultures was able to suppress gamma-herpesvirus lytic replication *in vitro*.

We therefore stimulated the latently EBV-infected BL cell line Akata with increasing concentrations of hemozoin or the synthetic TLR9 ligand CpG as a positive control and subsequently induced reactivation by crosslinking the B-cell-receptor with anti-IgG antibodies as described before [20]. Six hours after cross-linking the BCR, we measured the expression level of EBV's master lytic regulator gene *BZLF1* [21] by quantitative polymerase chain reaction (qPCR) (Fig.1A). In untreated Akata cells, the expression of *BZLF1* was found to be below detection level, and the addition of TLR9 ligand CpG did not change that. Treatment of Akata cells with anti-IgG antibody resulted in a strong increase of *BZLF1* expression, indicating the induction of lytic EBV replication. Pretreatment with CpG diminished the induction of *BZLF1* expression to about 20%. Interestingly, at higher concentrations, pretreatment with natural hemozoin hindered induction of lytic EBV gene expression in a dose-dependent manner.

Next, we tested the impact of hemozoin on another gamma-herpesvirus, the murine pathogen murine herpesvirus 68 (MHV-68). Latently MHV-68-infected S11 cells were treated with increasing concentrations of hemozoin or mouse-optimized CpG ODN 1826 as a positive control. 24 hours after stimulation, expression of the master lytic regulator gene of MHV-68, *ORF50*, was measured by qPCR (Fig.1B). Since spontaneous reactivation events are much more frequent in this cell line than in Akata, induction of the lytic cycle was not necessary for these experiments to readily detect expression of *ORF50*. Similarly to the previous results in Akata cells, treatment with hemozoin decreased the expression of *ORF50* in a dose-dependent manner.

To check whether hemozoin blocks induced gamma-herpesvirus reactivation analogous to the experiments in Akata, we incubated MHV-68-infected murine B-cell line A20HE1 with natural hemozoin and subsequently tried to reactivate MHV-68 by addition of Tetradecanoylphorbol acetate (TPA), an activator of gamma-herpesvirus lytic replication [22]. As expected, addition of TPA alone increased *ORF50* expression 8-fold (Fig.1C).

However, if the cells were preincubated with the natural hemozoin extract, the induction of lytic MHV-68 gene expression was substantially weakened.
These experiments showed that a hemozoin extract from *P. falciparum* cultures is able to suppress both spontaneous as well as induced reactivation of lytic replication in two species of gamma-herpesviruses.

Synthetic hemozoin can be polymerized from hemin and is indistinguishable from the natural hemozoin by infrared spectroscopy

Our first series of experiments was conducted with hemozoin extracted from *P. falciparum* cultures by using its magnetic properties. Since there are contradictory reports whether the hemozoin itself or rather contamination by parasite DNA or other products are the stimulating agent [19], we next asked if a synthetic hemozoin-like crystal can reproduce the repressive effect on gamma-herpesvirus replication that we observed with both hemozoin and CpG.

The synthetic hemozoin, called β-hematin, was polymerized from porcine hemin by a method adapted from Jaramillo et al [17]. The resulting product was analyzed by infrared spectroscopy (Fig.2). The spectrum showed three major peaks characteristic for β-hematin at 1712 cm^{-1}, 1662 cm^{-1} and 1210 cm^{-1}. Comparison with IR-spectra of synthetic β-hematin synthesized by other groups (Fig.2, small panel) or natural hemozoin confirmed composition and strucure of the compound (Fig. 2).

Synthetic hemozoin compound does not suppress gamma-herpesvirus lytic reactivation

To test whether the crystalline structure itself or possible contaminants in the natural hemozoin extract are reponsible for the effect observed in Fig.1, we repeated the experiments with newly synthesized hemozoin. First, we treated Akata cells with increasing concentrations of synthetic hemozoin (sHz) or TLR9 ligand CpG and after 2h induced lytic replication by IgG crosslinking. While CpG treatment reduced *BZLF1* expression to the same extent as before, no reduction in lytic EBV gene expression was observed after preincubation with sHz (Fig.3A). In the murine cell line S11 harbouring MHV-68, we also did not observe changes in lytic MHV-68 gene expression upon stimulation of the cells with sHz (Fig.3B).

Addition of P.falciparum DNA does not restore the effect of synthetic Hz on MHV-68 lytic replication

Parroche et al. [19] investigated the immunogenicity of both natural hemozoin and its counterpart β-hematin and came to the conclusion that β-hematin was immunologically inert.

They further reported that the stimulatory effect of natural hemozoin is caused by *Plasmodium* DNA bound to its surface. We wanted to test whether the addition of *Plasmodium* DNA to the synthetic hemozoin might restore the effect of natural hemozoin on MHV-68 gene expression. We mixed sHz with *Plasmodium* DNA at varying concentrations and used the mixtures to stimulate S11 cells (Fig.4). sHz or *Plasmodium* DNA alone did not show any effect on *ORF50* expression and neither did the combinations of the two.

Discussion

Hemozoin accumulates in parasitized red blood cells during *P. falciparum* infection, is subsequently released into the bloodstream, and has been claimed to be a ligand for TLR9. There is a controversy whether synthetically produced hemozoin itself binds to TLR9 or if parasite DNA bound to the crystal is responsible for the observed activation of TLR signaling. This is noteworthy since stimulation of TLR9 has a suppressive effect on gamma-herpesvirus lytic gene expression *in vitro* and leads to an increase in latently infected cells *in vivo*, similar to clinical observations in chronically malaria-infected patients. Therefore, we assessed the ability of natural hemozoin from *P. falciparum* cultures as well as synthetic β-hematin to suppress gamma-herpesviral lytic gene expression *in vitro*. We found that i) hemozoin extracts from *Plasmodium* culture downregulate the expression of lytic genes of two species of gamma-herpesviruses in a dose-dependent manner; ii) the synthetic hemozoin analogue β-hematin does not change the expression of gamma-herpesvirus lytic genes; and iii) the addition of *Plasmodium* DNA to the β-hematin preparation did not restore the reactivation-suppressing activity of hemozoin.

In Burkitt's lymphoma cells Akata that are latently infected with EBV, increasing concentrations of natural hemozoin led to a reduction in viral lytic gene expression after cross-linking of the B-cell receptor. The observed suppression was not as strong as with the synthetic ligand CpG, but nevertheless significant at higher concentrations (Fig.1A). In S11 cells infected with MHV-68 we showed a suppression of spontaneous lytic gene expression at a level close to that observed after treatment with CpG (Fig.1B). These results provide evidence for the ability of crude hemozoin extract to suppress reactivation in two gamma-herpesvirus species reminiscent of the effect of TLR9 stimulation. Hemozoin was shown before to stimulate dendritic cells in a TLR9- and MyD88-dependent fashion [14,15]. Thus, it is reasonable to assume that the reactivation-suppressing effect stems from activation of TLR9 signaling. However, from our data we cannot exclude that other immune receptors are involved in this phenomenon as the crude hemozoin preparation might contain other

stimulatory agents such glycosylphosphatidylinositol (GPI) which was shown to activate TLR2 signaling [23] as well as *Plasmodium*-derived hypoxanthine/xanthine which is degraded to uric acid after red blood cell rupture and activates endosomal receptors such as NALP3 inflammasome [24].

To exclude stimulatory effects of unknown contaminants in our hemozoin preparation, we synthesized β-hematin from HPLC-purified hemin and confirmed the composition and structure by infrared spectroscopy. When we stimulated herpesvirus-infected cells with β-hematin, we did not observe any change in lytic viral gene expression. This is in line with the results of Parroche et al. who found β-hematin to be immunologically inert and identified *Plasmodium* DNA associated with natural hemozoin as the active ligand for TLR9 [19]. On the other hand, there are several reports where β-hematin was found to activate TLR9 signaling in dendritic cells and macrophages [17,18] and binding studies demonstrating the binding of β-hematin to TLR9 by circular dicroism spectrum analysis [25]. A potential explanation for this apparent contradiction was offered by Jaramillo et al. who found that the size of the β-hematin crystals varied greatly depending on the source of hemin and the method employed in synthesis and showed that the immunogenicity of the product is largely dependent on the size distribution of the particles. Since the β-hematin crystals are indistinguishable in their infrared spectrum irrespectable of size, we were not able to further determine the precise morphology of our crystals.

According to Parroche et al. hemozoin acts as a vehicle for plasmodial DNA and facilitates the uptake into endsomes and presentation to TLR9. However, when we mixed β-hematin with varying amounts of *Plasmodium* DNA, we were not able to reproduce the reactivation-suppressing effect of the natural hemozoin extract. However, as we did not control for the attachment of DNA to the β-hematin or the efficient uptake into cellular endosomes, we cannot conclusively state that such a process would not result in the stimulation of TLR9 and therefore suppress gamma-herpesvirus reactivation.

Taken together, our results show that natural hemozoin extracted from *P. falciparum* culture leads to a reduction in gamma-herpesvirus lytic gene expression *in vitro* that is likely mediated by TLR9 signaling. In a mouse model, we showed before that constant stimulation of TLRs during persistent gamma-herpesvirus infection *in vivo* increases the number of latently infected B-cells in a similar fashion as seen for latent EBV infection in patients from regions with holoendemic malaria. It is therefore conceivable that the constant immune activation caused by malaria is an important factor in the etiology of eBls and that hemozoin is a possible mediator. However, we have to consider that constant immune stimulation is

probably not the only reason for a persistent EBV infection to develop into eBL. Another important factor might be that *P. falciparum* interferes with the host immune system and therefore weakens immune control of latent EBV infection. Several studies have shown that malaria leads to an impairement of EBV-specific $CD8^+$ T-cell responses [26-29], eventhough the exact mechanism is unclear. Furthermore, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) expressed on infected erythrocytes was reported to interfere with dendritic cell maturation, therefore hampering induction of an efficient cellular immune response [30].

Material and Methods

Cell culture. Human Burkitt's Lymphoma cell line Akata (courtesy of Dr. A. Bell, Birmingham, UK) and MHV-68-infected murine B-cell line S11 (courtesy of Prof. Ren Sun, Los Angeles, CA) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% penicillin-streptomycin and 2mM L-glutamine; (all reagents from Invitrogen, Basel, Switzerland) at 37°C and 5% CO_2 humidified atmosphere.

Natural hemozoin extract. Natural hemozoin extract was prepared as described by Parroche et al [19]. Erythrocytic stages of *P. falciparum* laboratory strain 3D7 were washed in PBS and resuspended at a hematocrit of ≈ 2%. RBC suspension was loaded onto an LS column (Miltenyi Biotec, Bergisch Gladbach, Germany) and placed in the MACS separator. The magnetic field allowed the hemozoin to be retained due to its magnetic properties, while the rest of the culture flowed through. The column was washed with PBS, removed from the magnetic field, and the hemozoin was eluted and quantified.

Hemozoin quantitation. To quantify natural hemozoin extract, total heme content was determined as described before [31]. Hemozoin was depolymerized for 2 hours at room temperature in 20mM NaOH and 2% SDS. OD was read at 400nm using NanoDrop ND-1000 Spectrophotometer (Witec AG, Luzern, Switzerland). 25µg of hemozoin or β-hematin contains 26-29 nmol of heme.

Reverse transcription and quantitative PCR. Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland) and contaminating DNA was removed by DNAse treatment (DNA-free Ambion; Applied Biosystems, Rotkreuz, Switzerland). cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Amplification of synthesized cDNA was performed using TaqMan®

Gene Expression Master Mix (Applied Biosystems). For EBV lytic gene *BZLF1* the following primers and probe were used: BZLF1_forward 5'-CACGACGTACAAGGAAAC-3'; BZLF1_reverse 5'-CGCTTTATTTCTAGTTCAGAATCGC-3'; BZLF1_probe 5'-FAM-CAGCCAGAATCGCTGGAGGAA-BHQ-1-3'. For MHV-68 lytic gene ORF50 the following primers and probe were used: ORF50_forward 5'-CCAACGTGTTCCCAGAAC-3'; ORF50-reverse 5'-CGATGAACGCGTCCTCAG-3'; ORF50_probe 5'-FAM-TACTCAGGAAGCGTGTCCCGGATCA-BHQ-1. All reactions were run on ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Rotkreuz, Switzerland). Amplification of *gapdh* mRNA was used for normalization within each sample.

Synthesis of β-hematin. β-hematin was produced by the aqueous acid-catalyzed method as proposed by Jaramillo et al [17]. 500mg of HPLC-purified hemin (Sigma-Aldrich, Buchs, Switzerland) was dissolved in 100ml degassed 0.1M NaOH under gentle stirring. Propionic acid was added dropwise until pH reached 4. The mixture was heated in a waterbath at 70°C for 18 hours, cooled down and then filtered. The product was scratched from the filter and washed in 100ml 0.1M $NaHCO_3$ for 3 hours. The filtrate was then washed 3 times alternatingly in MilliQ H_2O and Methanol before being dried over night in a vaccuum oven. The resulting product was analyzed in a infrared spectrometer.

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Figure 1

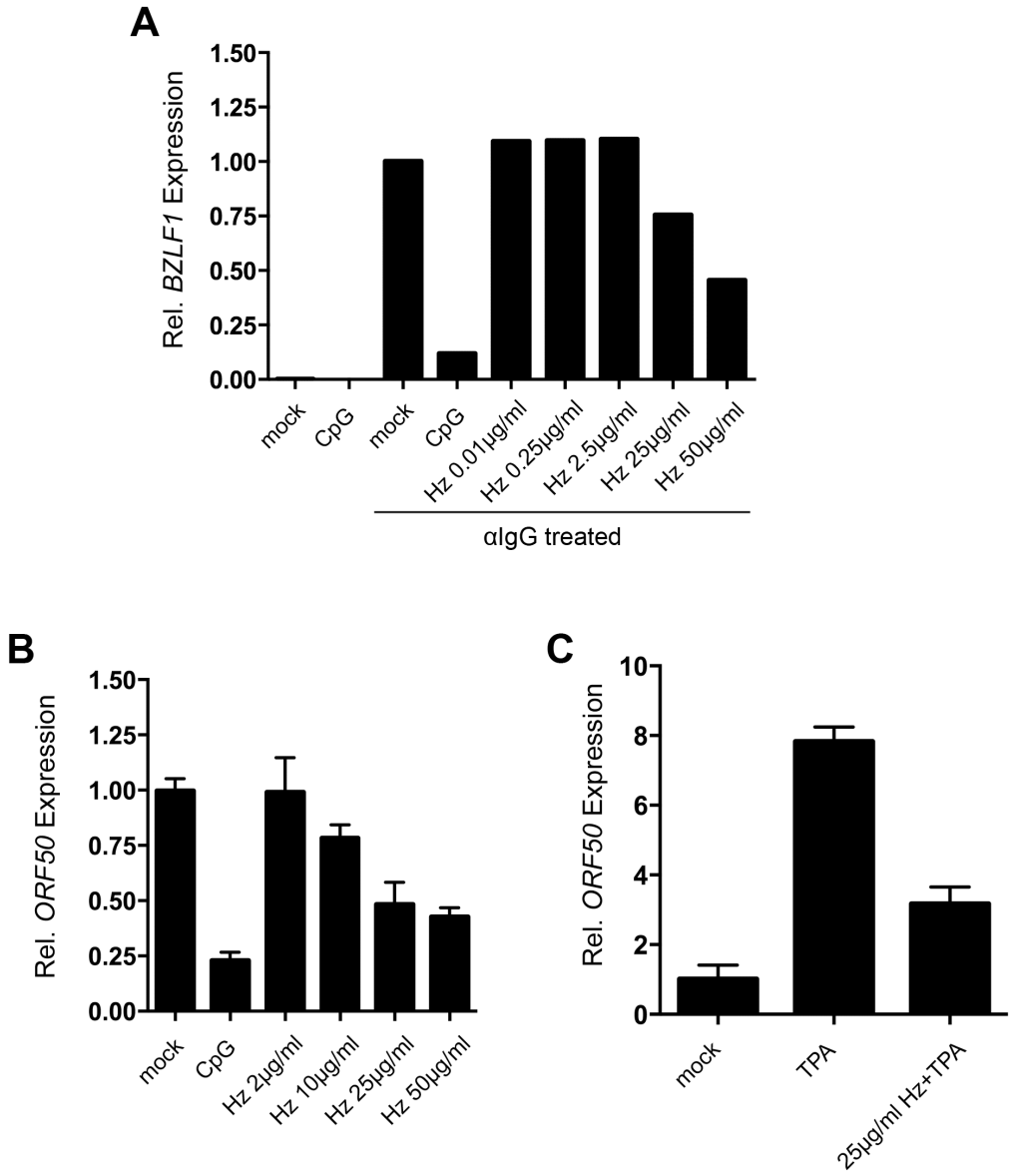
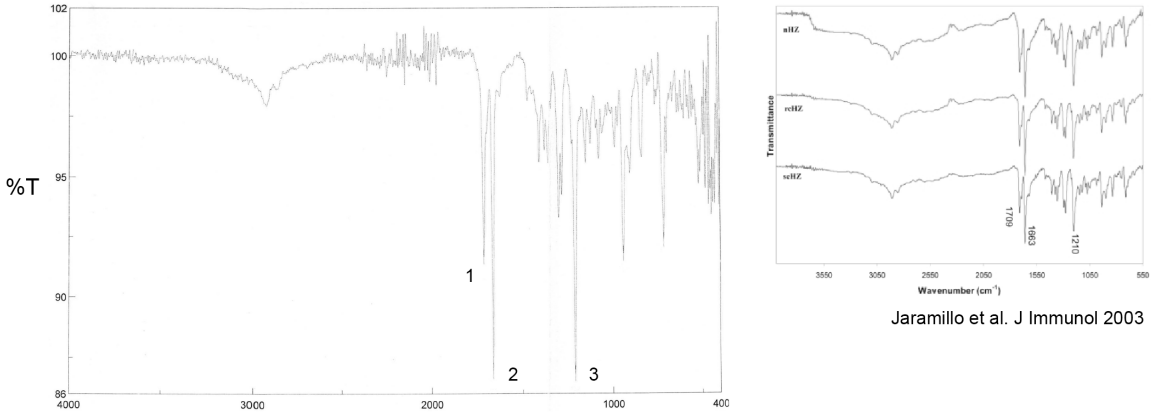


Figure 1. Hemozoin extract from Plasmodium falciparum culture downregulates spontaneous and induced expression of gamma-herpesvirus lytic genes in a dose-dependent manner in vitro.
(A) Latently EBV-infected Burkitt's lymphoma cells Akata were treated with 0.5µM CpG ODN 2006 or hemozoin at various concentrations 2h before induction of reactivation by treatment with 100µg/ml anti-IgG. 6 hours after induction, cells were harvested and the expression of EBV lytic gene BZLF1 was measured by quantitative PCR and normalized to the human housekeeping gene HMBS.
(B) MHV68-infected murine B-cells S11 were treated with 0.5µM CpG ODN 1826 or hemozoin at various concentrations. 24 hours thereafter, cells were harvested and the expression of MHV-68 lytic gene ORF50 was measured by quantitative PCR and normalized to the murine housekeeping gene gapdh.
(C) MHV-68-infected murine B-cells A20HE1 were treated with 25µg/ml hemozoin 2h before inducing reactivation with 10ng/ml TPA. 24 hours after induction, cells were harvested and ORF50 expression was measured as in (B).

Figure 2



Peak Picking:

Nr.	Position	Intensity	Nr.	Position	Intensity	Nr.	Position	Intensity
1	1712.48	91.67	2	1662.34	86.92	3	1210.11	86.83

Figure 2. β-hematin (synthetic hemozoin) synthesized from hemin is pure and indistinguishable from natural hemozoin by infrared spectroscopy. β-hematin was analysed by infrared spectroscopy (big panel). Characteristic peaks were compared to natural hemozoin and β-hematin from other sources (small panel, from Jaramillo et al, J Immunol 2003).

Figure 3

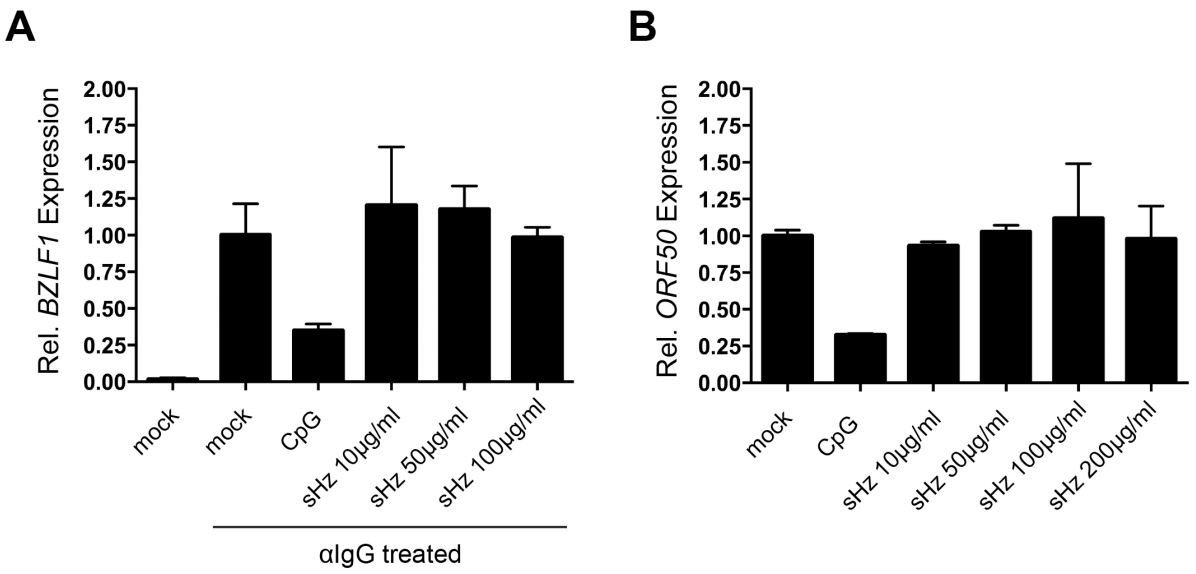


Figure 3. β-hematin does not affect the expression of gamma-herpesvirus lytic genes in vitro.
(A) Akata cells were treated with 0.5µM CpG ODN 2006 or β-hematin (sHz) at various concentrations 2h before induction of reactivation by treatment with 100µg/ml anti-IgG. 6 hours after induction, cells were harvested and the expression of EBV lytic gene BZLF1 was measured by quantitative PCR as described before.
(B) S11 cells were treated with 0.5µM CpG ODN 1826 or β-hematin (sHz) at various concentrations. 24 hours after treatment, cells were harvested and the expression of MHV-68 lytic gene ORF50 was measured by quantitative PCR as described before.

Figure 4

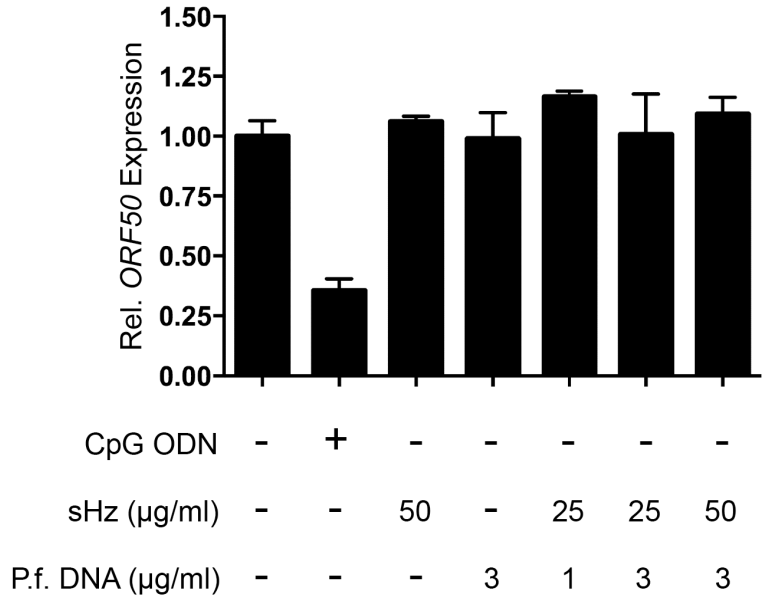


Figure 4. Plasmodium falciparum DNA alone or in combination with β-hematin does not affect gamma-herpesvirus lytic gene expression. (A) S11 cells were treated with either 0.5µM CpG ODN 1826, β-hematin (sHz), P. falciparum DNA or combinations of β-hematin and P. falciparum DNA. 24 hours after treatment, cells were harvested and the expression of MHV68 lytic gene ORF50 was measured by quantitative PCR as described before.